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UNIVERSITY OF ALBERTA

STEREOCHEMISTRY OF THE BOVINE AND PORCINE SEMICARBAZIDE SENSITIVE AMINE OXIDASE REACTION

by

Christine Holly Scaman

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

Doctor of Philosophy

IN

Food Chemistry

Department of Food Science

EDMONTON, ALBERTA

Fall 1991

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ISBN 0-315-69973-6

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NAME OF AUTHOR

Chrisitine Holly Scaman

TITLE OF THESIS

STEREOCHEMISTRY OF THE BOVINE AND PORCINE SEMICARBAZIDE SENSITIVE AMINE OXIDASE REACTION

DEGREE FOR WHICH THESIS WAS PRESENTED **Doctor of Philosophy**

YEAR THIS DEGREE GRANTED **Fall 1991**

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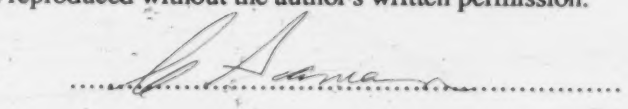

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis

entitled **STEREOCHEMISTRY OF THE BOVINE AND PORCINE SEMICARBAZIDE SENSITIVE AMINE OXIDASE REACTION**

submitted by **Christine Holly Scaman**

in partial fulfillment of the requirements for the

degree of **Doctor of Philosophy**

in **Food Chemistry**

Monica Palcic
M. M. Palcic, Supervisor

H. Jackson

L. Ozimek

Peter Sporns
P. Sporns

J. Vederas

Peter Sporns
M. Gelb (External Examiner)

Date: Sept 21/94

DEDICATION

To

Mom & Dad

Abstract

Amine oxidases catalyze the deamination of amines using molecular oxygen as the terminal electron acceptor and thus produce an aldehyde, hydrogen peroxide and ammonia. The stereochemical course of amine oxidation by two semicarbazide sensitive amine oxidases (SSAO) from bovine and porcine aortic tissue (no E.C. designation), were compared to that of two copper amine oxidases from sheep plasma and porcine kidney (E.C.1.4.3.6).

The reactivity of the SSAO with semicarbazide and phenylhydrazine suggested that the cofactor was a quinone type molecule with properties distinct from TOPA (2,4,5-trihydroxyphenylalanine), the cofactor of some copper amine oxidases. The oxidation of tyramine was found to proceed with loss of the pro-*S* proton at C-1, coupled with solvent exchange into C-2, a pattern which has not been observed for any copper amine oxidases examined to date. The solvent exchange reaction also occurred stereospecifically, with the loss from, and reprotonation to, the pro-*R* position. This suggests that these two processes occur from the same face of the enamine double bond. The stereochemical patterns observed, coupled with a preliminary physio-chemical characterization of the SSAO suggests that these enzymes may constitute a class of amine oxidase distinct from the copper amine oxidases.

Proton abstraction from C-1 of tyramine catalyzed by the porcine kidney amine oxidase also occurred stereospecifically with the loss of the pro-*S* proton. However, the solvent exchange pathway was not exhibited. This stereochemical pattern is consistent with a subsection of copper amine oxidases, including those from plant seedlings, best described as diamine oxidases, and suggests that the porcine kidney amine oxidase is most closely related, mechanistically, to the plant seedling type amine oxidases.

The sheep plasma amine oxidase was purified to a higher specific activity (0.13 U/ mg protein) than previously obtained. Reactivity of the cofactor of the enzyme with phenylhydrazine indicated that it may be TOPA. Using the high specific activity enzyme, an apparent nonstereospecific proton abstraction at C-1 of tyramine, characterized by dual binding modes, was confirmed as a property intrinsic to the enzyme. The enzyme catalyzed the solvent exchange reaction with nonstereospecific abstraction of a proton from C-2, followed by reprotonation from solvent to the pro-*R* position. This stereochemical pattern of proton abstraction from C-1 and C-2 is very similar to that previously observed for the bovine plasma amine oxidase, suggesting by analogy, that it may by catalyzed by a single base.

Acknowledgements

I would like to express my gratitude to the people who were involved in some aspect of my work. My supervisor, Monica Palcic, provided guidance, ideas, and enthusiasm throughout this project, and helped to expand my interest in science.

I would like to acknowledge Dr. Ole Hindsgaul for his consultation on the NMR work and for the chemical conversion protocol "that even a food scientist could follow" . The suggestion of Dr. John Beale to use a chiral derivatizing agent proved to be very useful, and was greatly appreciated. I would also like to thank my committee members for their constructive criticism of my thesis.

The discussions and fun shared with Araba, Gord, Lahku, Liz, Louis, Suzanne and Young made the lab a genial place to work. A note of thanks is due to Suzanne and Liz for helping to run numerous NMR samples, and to Adam, for doing the photographic work.

Finally, I would like to acknowledge the support and encouragement I received from my family and from my husband, David, whose positive outlook gave me perspective during the periods of frustration that accompany any research, and who shared the good times with me.

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List of Abbreviations

CHAPS	(3-(3-cholamidopropyl)dimethyl-ammonio-1-propanesulfonate)
Con-A	Concanavalin A
DBU	1,8-diazabicyclo-[5.4.0]undecene-7
DCC	N,N'-dicyclohexylcarbodiimide
DMAP	4-dimethylaminopyridine
DMF	N,N-dimethylformamide
DEAE	diethylaminoethyl
E.C.	Enzyme Commission
EDTA	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
HTP	hydroxyapatite
Km	Michaelis constant
MAO	monoamine oxidase
MHz	megahertz
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NADH	nicotinamide adenine dinucleotide, reduced form
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
ppm	parts per million
PLP	pyridoxal phosphate
PQQ	pyrroloquinoline quinone
Rf	relative mobility
SDS	sodium dodecyl sulfate
SSAO	semicarbazide sensitive amine oxidase
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
TOPA	2,4,5-trihydroxyphenylalanine
TRIS	tris(hydroxymethyl)methylamine
U	international units of enzyme activity

Chapter 1. Classification and General Characteristics of Amine Oxidases

Amine oxidases catalyze the oxidative deamination of amines, with the concomitant production of an aldehyde, hydrogen peroxide and ammonia (Equation 1.1).

$$R\text{-}CH_2NH_2 + O_2 + H_2O \rightarrow R\text{-}CH{=}O + H_2O_2 + NH_3 \qquad (1.1)$$

They constitute a very diverse group of enzymes that are widely distributed in organisms. Activity has been reported in bacteria, fungi, yeast, plants and sundry animal tissues (1). No classification scheme can as yet account for all the activities that have been described, although attempts have been made to categorize the enzymes based on substrate specificity, cofactor requirements, source of the enzyme, or sensitivity to inhibitors. This classification has evolved as more detailed information has become available concerning the physio-chemical and mechanistic properties of the enzymes.

The first report of an amine oxidase was that of rabbit liver (2), followed soon afterwards by a report of an enzyme from lung which deaminated histamine (3). Differences in the properties of the two enzymes resulted in the former enzyme being referred to as a monoamine oxidase (MAO) while the latter was defined as a diamine oxidase (DAO) (4). Later it was recognized that two forms of the enzyme, MAO-A and MAO-B, contained a flavin cofactor (5), and these have remained a distinct group with an Enzyme Commission (E.C.) classification of 1.4.3.4. These enzymes are mainly localized in the outer mitochondrial membrane of animal cells (6) and act on neuroactive amines (7). There has been considerable pharmacological and mechanistic interest in these enzymes since the fortuitous discovery that their inhibition relieved symptoms of depression (8). More recently, interest has also been heightened as the metabolism of the drug MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) by MAO-B has been found to cause a syndrome similar to Parkinson's disease in humans (9).

The classification of diamine oxidases (E.C. 1.4.3.6) encompasses a variety of enzymes, some of which act optimally on diamines such as putrescine and cadaverine, while others show a decided preference for monoamines such as benzylamine. In general, these enzymes display rather broad substrate specificity, and this is reflected by a low turnover rate (10). All these enzymes are sensitive to carbonyl reagents such as semicarbazide and phenylhydrazine, suggesting that they utilize a non-flavin, carbonyl cofactor (11).

In addition to the organic cofactor, the enzymes have 2 atoms of copper (Cu^{+2}) per monomer of enzyme (12) and are referred to as the copper containing amine oxidases. The copper is believed to function in the electron transfer mechanism (13), and recently evidence for the formation of a Cu^{+1}-semiquinone under anaerobic conditions was obtained (14). Examples of these copper amine oxidases include those found in the plasma of ruminant and nonruminant animals, tissues including the kidney, liver and intestine, and plant seedlings (15). These copper containing enzymes are typified by having a peach or pink colour in a concentrated solution or in crystals (16), thought to be due to the nature of the organic cofactor.

The terms benzylamine oxidase and semicarbazide sensitive amine oxidase have been used liberally in the past literature to refer to various members of the non-flavin amine oxidases, when no clear-cut mechanistic basis exists for either classification. In this work, the term semicarbazide sensitive amine oxidases is used only to refer to a group of enzymes which have not yet been assigned an E.C. classification. Two members from this group, isolated from bovine and porcine aortic tissue, are the focus of the present study. Characterization of the enzymes in this work and of related enzymes in the literature indicate that the term diamine oxidase is not appropriate as the monoamine, benzylamine, is the preferred substrate and diamine oxidase activity is very low (17). As well, a dependence on copper for catalytic activity has not been demonstrated (18), precluding the term copper-dependent or copper-containing amine oxidase. The enzymes are, however, inhibited by the carbonyl reagent semicarbazide, similar to the copper amine oxidases, although the nature of the cofactor has not been established (19).

The semicarbazide sensitive enzymes are distinguished from the copper containing diamine oxidases by their high affinity for benzylamine. This amine does not occur physiologically, however, and the true substrate of the enzyme is unknown (19). It has been suggested that methylamine, a byproduct of bacterial or endogenous metabolism of creatinine, lecithin, sarcosine or choline, may be one substrate of the enzyme *in vivo* (20). In addition, the enzymes tend to be membrane bound (21) unlike the soluble copper amine oxidases. Vascular tissue is a particularly good source of the enzyme, and it is suggested that the enzymes play a yet undefined role in the maintenance of cardiovascular function (22). Their activity, though, has been associated with the formation of cardiovascular lessions, through the deamination of allylamine to acrolein (23). The localization of the enzyme in the plasma membrane was suggested to indicate that a product of the reaction may act as a transmembrane signal (19).

Despite distinct differences, the semicarbazide sensitive enzymes have some physio-chemical features in common with the copper amine oxidases. As noted above,

both types of enzyme are inhibited by semicarbazide indicating that the cofactor of each is a carbonyl compound. Members of both groups appear to be dimers, with native molecular weights usually ranging from 170 to 196 kDa (24, 25), although copper amine oxidase from *Aspergillus niger* is reported to be 252 to 273 kDa (15) while that from *Hansenula polymorpha* is reported to be 79 kDa (26). As well, both types of amine oxidases are glycoproteins, and display affinity for concanavalin A and *Lens culinaris* lectin (13, 19).

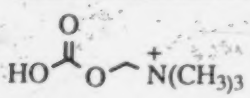
The wide distribution of the non-flavin amine oxidases suggests they play an important role in the metabolism of biogenic amines, which arise from endogenous amino acid metabolism (27). The role for these enzymes has often been assumed from the localization of activity. For example, the pig kidney diamine oxidase is localized in the brush border and tubule epithelium suggesting a role as an amine scavenger (28). A similar role has been suggested for the plasma amine oxidase (29). The detoxification of exogenous amines, arising from the ingestion of fermented foods, has also been suggested as a physiological role for the enzymes (30).

Amines themselves, are implicated in critical physiological roles (Table 1.1). Dopamine, serotonin, acetylcholine, epinephrine and norepinephrine function as neurotransmitters, and regulation of their activity can be partially controlled through degradative processes (30). In plants, the oxidation of amines is a key step in the biosynthesis of alkaloids (31). Similarly, polyamines, such as spermine and spermidine, have been associated with the sensitive processes of cell growth and differentiation (32). Amine oxidases may control the level of polyamines by acting directly on the polyamines or their precursor, putrescine.

Despite the interest in the non-flavin enzymes based on their potential physiological impact, the mechanism of these enzymes is not well understood. In part, this is due to the lack of an amino acid sequence and crystal structure for the enzymes, although an amino acid sequence for an amine oxidase from the yeast *Hansenula polymorpha* was recently determined from the gene encoding the protein (26). In addition there has been uncertainty about the structure of the cofactor. Two compounds previously believed to be the cofactor of the copper containing plasma amine oxidases, and the compound recently unambigously identified as the cofactor are given in Figure 1.1. The well known sensitivity of the enzymes to carbonyl reagents was taken in the older literature as support for the presence of pyridoxal phosphate (33). However, direct evidence for the presence of pyridoxal phosphate was not procured and arguments for and against it as the cofactor were made up until 1990. Two independent reports in 1984, based on indirect evidence, suggested that pyrroloquinoline quinone (PQQ) was the cofactor of the copper amine oxidase from bovine plasma (34, 35). These reports were viewed with considerable interest as PQQ possibly

Table 1.1. Physiological Roles of Amines and Amine Moieties

Neurotransmitters and Hormones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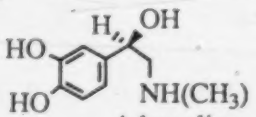
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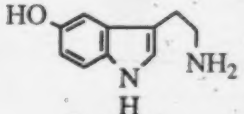
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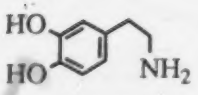
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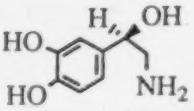
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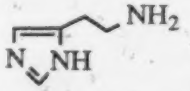
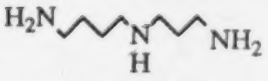

Histamine

Cell Growth / Differentiation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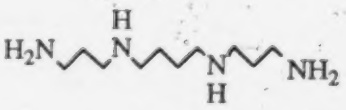
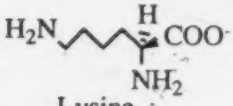
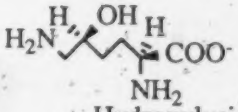

Spermine

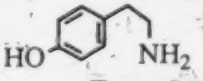
Peptidyl Cross-Linking


Lysine

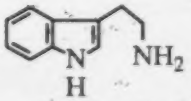

Hydroxylysine

Precursors of Polyamines / Alkaloids

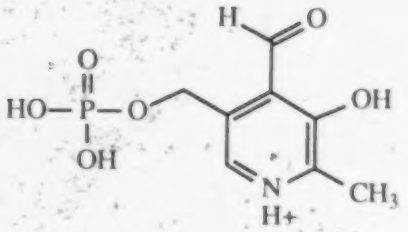
Putrescine


Tyramine

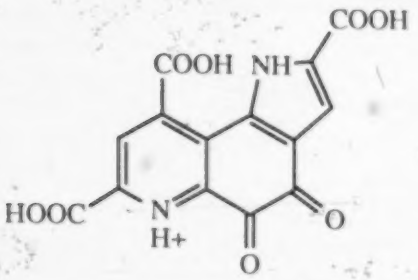
Cadaverine


Tryptamine

A. Pyridoxal Phosphate (PLP)



B. Pyrroloquinoline Quinone, oxidized form (PQQ)



C. 2,4,5,-Trihydroxyphenylalanine, oxidized form (TOPA)

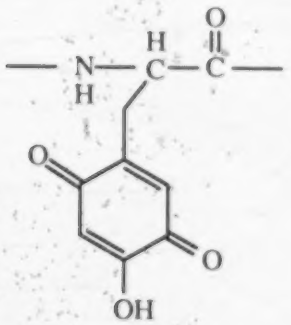
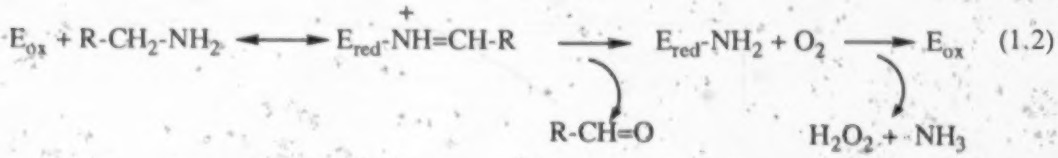


Figure 1.1. Structure of cofactors previously believed to be present in some copper amine oxidases (A & B), and the true cofactor of bovine plasma amine oxidase (C).

represented the first new cofactor, and potential vitamin, of animal metabolism to be discovered in recent years. Mechanistic data available for the copper amine oxidase could be reconciled with the presence of PQQ in the active site (36), and a rash of reports suggesting the presence of PQQ in other oxidases, including lysyl oxidase (37), pig kidney diamine oxidase (38), methylamine oxidase (39) and pea seedling amine oxidase (40) appeared. However, doubts concerning the methodology used to detect PQQ arose when crystallographic densities for methylamine dehydrogenase, a supposed PQQ-containing enzyme, could not be reconciled with the structure of PQQ (41). Recently, conclusive identification of the cofactor of bovine plasma amine oxidase as 2,4,5-trihydroxyphenylalanine, or TOPA was obtained (42). Additional evidence suggested that TOPA is also present in several other copper amine oxidases (43, 44). However, the identification of the cofactor of the semicarbazide sensitive amine oxidases has not been established. Preliminary characterization of the cofactor of aortic semicarbazide sensitive amine oxidase in this work suggests it is not TOPA.

Mechanistically, the amine oxidases from bovine and porcine plasma are the best characterized of the non-flavin enzymes. It was recently shown that the bovine plasma enzyme follows a transaminase type of mechanism as aldehyde is released anaerobically without the release of ammonia (43) (Equation 1.2).



(1.2)

Reports on the pH dependence of proton abstraction from C-1 and C-2 of dopamine by the bovine plasma amine oxidase indicate that the process is enzyme-mediated through a basic residue (45) which has not yet been identified. An analysis of the stereochemical course of the bovine plasma amine oxidase reaction also supports the proposal that a single base is involved in catalysis (46).

Stereochemical studies have also shown that the bovine plasma enzyme exhibits dual binding modes for some substrates (47). The enzyme displays an unusual substrate dependent stereochemistry as benzylamine is oxidized via abstraction of the pro-*S*[1] proton at C-1 (48) while dopamine (47) and tyramine (49) are oxidized with an apparent

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[1] The stereochemical terminology of pro-S (or pro-R) refers to the proton of a pro-chiral methylene which if given higher priority in the Cahn-Ingold-Prelog system, generates a chiral center with an S (or R) configuration.

nonstereospecificity. The apparent nonstereospecificity has been attributed to opposite but stereospecific binding modes for these substrates. The pea seedling amine oxidase apparently lacks the ability to bind some substrates in alternate modes as benzylamine (31), tyramine (49) and dopamine (47) are oxidized by abstraction of only the pro-*S* proton at C-1. Interestingly, the stereochemistry of the porcine plasma amine oxidase reaction with dopamine and tyramine occurred with abstraction of the pro-*R* proton at C-1 (49). This then, represents the first formal class of enzymes in which a reaction is catalyzed by all possible stereochemical modes (pro-*R*, pro-*S* and nonstereospecific).

To further investigate the relationship between the copper amine oxidases and the semicarbazide sensitive amine oxidases, the stereochemical mode of tyramine oxidation by the bovine and porcine aortic semicarbazide sensitive enzymes were compared to that of two copper amine oxidases, from porcine kidney and from sheep plasma. The stereochemistry of substrate oxidation by the bovine and porcine aortic enzymes has not been reported, although the reaction catalyzed by an enzyme from rat aorta was reported to proceed with pro-*S* proton abstraction at C-1 of dopamine (50) and benzylamine (51). Subsequent chapters will report on (i) the results of the isolation and partial characterization of the enzymes used in the study, (ii) the stereochemistry of proton abstraction at C-1 of tyramine, (iii) the stereochemistry of the proton abstraction at C-2 and (iv) the mechanistic implications of the stereochemical mode of reaction of the semicarbazide sensitive amine oxidases compared to the copper amine oxidases.

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Chapter 2. Isolation of Semicarbazide Sensitive and Copper Containing Amine Oxidases and Partial Charaterization

Introduction

A review of the classifications and general characteristics of amine oxidases was reported in Chapter 1. In this section, an overview of methods used in the isolation of three amine oxidases of interest are reported. These published methods provided the basis for the isolation protocols of bovine and porcine aortic semicarbazide sensitive amine oxidase, porcine kidney amine oxidase and sheep plasma amine oxidase used in this work. In addition, an initial characterization of the properties of semicarbazide sensitive amine oxidase, including a preliminary examination of the cofactor of this enzyme will be reported.

Amine oxidases that are inhibited by semicarbazide are found in a wide variety of animal tissues (1). They encompass the copper containing amine oxidases, such as the plasma amine oxidases and porcine kidney diamine oxidase which fall into the E.C. 1.4.3.6 classification. As well, this includes another group of enzymes in which copper dependence has not been shown and which have not been assigned an E.C. grouping. This second group is referred to as the semicarbazide sensitive amine oxidases (SSAO). These enzymes have been isolated from diverse sources including ox dental pulp (2), rat anococcygeus tissue (3), rat brown adipose tissue (4), horse liver (5), human umbilical artery (6), and rat aorta (7). Vascular tissue is a particularly good source of the enzyme (8) and the SSAO activity of aorta from several sources has been examined by researchers in the 1970's. In this work, bovine and porcine aortic tissue were chosen as sources of semicarbazide sensitive amine oxidase, since the availability and size of the tissue could potentially provide the amount of enzyme required for the physio-chemical and stereochemical experiments.

Aortic tissue contains a soluble and insoluble form of SSAO. O'Dell and coworkers (9) reported the 170 fold purification of a soluble bovine aorta amine oxidases which was sensitive to semicarbazide and less stable than the bovine plasma amine oxidase. Their preparation appeared to be a mixture of enzymes such as lysyl oxidase, polyamine oxidase and semicarbazide sensitive amine oxidase since lysyl-vasopressin activity was present and the polyamines spermine and spermidine were oxidized at rates greater than benzylamine. Following this, a Triton X-100 extractable, or insoluble, amine oxidase activity was obtained from bovine aorta and found to have characteristics unique from the soluble form of the enzyme (10). The soluble enzyme, which accounted for only 15% of the total activity, did not oxidize N-methylbenzylamine, tryptamine or histamine but was

active on polyamines. The insoluble enzyme did oxidize N-methylbenzylamine to a limited extent but was inactive on polyamines. While both enzymes were inhibited by semicarbazide, hydroxylamine and phenylhydrazine, the insoluble form was less sensitive to the first two reagents and more sensitive to the latter, when compared to the soluble activity. A soluble bovine aorta amine oxidase was purified 800 fold by DEAE-cellulose, hydroxylapatite, gel filtration, and affinity chromatography on Concanavalin A Sepharose 4B (11). Using this protocol, the amine oxidase activity was separated from contaminating lysyl oxidase activity and was shown to have properties distinct from the bovine plasma amine oxidase. This soluble enzyme was capable of oxidizing lysyl-vasopressin while the plasma enzyme was not. The soluble activity did not contain lysyl oxidase however, as it failed to act on other substrates of lysyl oxidase such as tropoelastin. A soluble benzylamine oxidase activity, inhibited by semicarbazide at 1 mM, was isolated from rabbit aorta (12). It was noted that only 20% of the activity of the total aorta homogenate could be obtained in the soluble fraction while the majority of the activity was retained in the tissue. As well, Buffoni and coworkers (13) isolated a soluble and insoluble amine oxidase activity from porcine aorta. They also showed that the porcine aorta contained an amine oxidase activity that was extractable by Triton X-100 but this activity was not characterized.

More recent attempts to purify SSAO have concentrated on the insoluble, or detergent extractable, form of the enzyme. The majority of SSAO activity appears to be localized on a cellular membrane as suggested by past findings that low amounts of activity were solubilized in buffers without detergents. This is supported by histochemical studies, where the enzyme has been shown to be associated with the plasma membrane in the rat aorta (14) and rat brown adipose tissue (15). SSAO was obtained from rat brown adipose tissue using Triton X-100 solubilization, gel filtration and affinity chromatography on Concanavalin A and *Lens culinaris* lectin (4). A preparation with a final specific activity of 0.04 units/mg protein was obtained when activity was assayed at 37°C. As well, SSAO was obtained from rat brown adipose tissue by binding cell membranes to polycationic beads (16). Cultured porcine aortic smooth muscle cells were used as a source of SSAO (17). Enzyme was obtained from cell homogenates by CHAPS (3-(3-cholamidopropyl)dimethyl-ammonio-1-propanesulfonate) extraction, followed by gravity and high performance anion exchange chromatography. A final specific activity of 0.07 units/mg protein was obtained when assayed at 37°C. The isolation protocol of porcine and bovine aortic semicarbazide sensitive amine oxidase used in this work was modelled on the techniques used for isolation of rat brown adipose tissue and cultured aortic smooth muscle cells described above.

The best substrate for SSAO, as determined by affinity and rate of catalysis, is benzylamine. This substrate, however, is not known to occur physiologically (5), and the biological role of the enzyme remains unclear. The enzyme catalyzes a typical amine oxidase reaction whereby an amine is deaminated in the presence of molecular oxygen to form an aldehyde, along with hydrogen peroxide and ammonia (Equation 2.1).

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCH{=}O + NH_3 + H_2O_2 \qquad (2.1)$$

Typically, SSAO displays a low Km value for benzylamine, although this varies with the source of the enzyme. For example, the Km value is 2.45 μM for rat brown adipose tissue enzyme (4), 1.7 μM for rat aorta enzyme (18), 5.1 μM for cultured porcine aorta smooth muscle cells (17), 161 μM for human umbilical artery (6), while values of 143 and 245 μM have been reported for the human aortic enzyme (19, 20). The enzyme from rat aorta is also active against a range of aliphatic amines from methyl to octadecylamine (21). Other aromatic amines such as histamine and β-phenethylamine are poorer substrates than benzylamine, as are the neurotransmitter amines noradrenaline and 5-hydroxytryptamine. The rat aorta SSAO displays some polyamine oxidase activity against spermine and spermidine with Km values of 2.9 and 1.9 mM respectively, but did not deaminate the diamines, cadaverine and putrescine.. Human umbilical artery SSAO was active at a reduced rate with aromatic amines such as tyramine and β-phenethylamine, which are relatively poor substrates with Km values of 17.6 and 13.3 mM respectively (6).

Little is known of the organic and inorganic cofactor requirements of SSAO. No evidence exists for the presence of copper, although a requirement has been suggested based on the similarities between known copper containing enzymes enzymes such as the plasma amine oxidase and SSAO (5). The rat brown adipose tissue enzyme is inhibited by cuprizone, a copper binding agent, but the interpretation of this information is complicated by the fact that cuprizone may also act as a carbonyl reagent. Other metal chelators such as EDTA and azide were reported to have no inhibitory effect on SSAO (15), so the copper dependence of these enzymes is unclear.

A distinctive feature of SSAO enzymes is their sensitivity to the carbonyl reagent semicarbazide, which has been characterized as being irreversible (22). The inhibition by carbonyl reagents suggests that the cofactor may be a quinone type molecule. SSAO from porcine dental pulp was inhibited 50% by a semicarbazide concentration of 130 μM (2) while porcine aortic smooth muscle cells were completely inhibited at 10 μM after 1 hour at 37°C (17). Only 5% of the initial activity remained in human umbilical artery after

incubating 20 minutes at 37°C at 100 μM semicarbazide (6) and the human aortic amine oxidase was inhibited > 90% at 1 mM concentration (1, 22). The inhibition by other carbonyl type reagents including hydroxylamine and phenylhydrazine has been demonstrated (2). Only recently has the identity of the cofactor of a number of copper containing enzymes been confirmed. Bovine plasma amine oxidase was shown to contain 2,4,5-trihydroxyphenylalanine, or TOPA (23). The cofactor structure was elucidated by forming a phenylhydrazone derivative, isolation of a small peptide containing the derivative, and identification by nuclear magnetic resonance and mass spectroscopy. The presence of TOPA in porcine plasma, porcine kidney and pea seedling amine oxidase was also strongly suggested, based on spectral properties of a phenylhydrazone derivative. This work ends decades of uncertainty regarding the cofactor in these enzymes. However, the nature of the cofactor in the semicarbazide sensitive amine oxidases remains to be determined.

In contrast to the relatively recent recognition and isolation of membrane bound SSAO, the purification of porcine kidney diamine oxidase (E.C.1.4.3.6) has been reported since 1943 (24). This enzyme is a copper containing amine oxidase which oxidatively deaminates aliphatic diamines with hydrocarbon chain lengths ranging from four to seven carbons. It also acts on histamine. The enzyme shows resistance to denaturation by heat and many isolation protocols include an incubation of crude fractions at 60°C for 10 minutes (25, 26). The enzyme was first obtained in a purified form by Mondovi and coworkers (27). Their isolation scheme included homogenization, heat treatment, ammonium sulfate precipitation, precipitation at pH 5.3, column electrophoresis, anion exchange chromatography and precipitation at 6.8. A 28% yield was obtained with a final specific activity of 1.27 units/mg protein[1]. As well, diamine oxidase has been obtained as homogeneous pink crystals (28). This procedure involved homogenization, ammonium sulfate precipitation, anion exchange chromatography, a second ammonium sulfate precipitation, chromatography on hydroxylapatite and gel filtration, and finally, crystallization. The final preparation had an activity of 1.08 units/mg protein and was obtained in 21% yield. An isolation protocol has been developed by Tamura and coworkers (29) starting from a lyophilized powder of diamine oxidase purchased from Sigma. This procedure included sequential chromatography on hydroxylapatite, DEAE-cellulose, and Con-A Sepharose. This preparation gave a high specific activity of 3.2

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[1] A unit = 1 μmole product / minute

units/mg protein when assayed with 3.3 mM putrescine at 30°C and a 25% yield. An unusual protocol for isolation of the diamine oxidase included a heat treatment, chromatofocusing and affinity chromatography using antibodies to the enzyme linked to Sepharose 4B (25). This preparation resulted in a final specific activity of only 0.1 units/mg protein when assayed at 37°C however, and more traditional techniques appear more suitable for obtaining an enzyme with a high specific activity. In the present study, a modification of the method of Tamura and coworkers (29) was used to obtain enzyme used for stereochemical studies.

Little is known about the sheep plasma amine oxidase. An enzyme which oxidized spermine was first reported to be present in sheep plasma in 1953 (30). Two isolation protocols have been developed. The isolation steps for the first included ammonium sulfate precipitation and extraction, adsorption of calcium phosphate gel and gel filtration resulting in a 36% yield and a final specific activity of 0.011 units/mg protein (31). This procedure was later modified to include an additional ammonium sulfate extraction and chromatography on a DE-52 column as a final step to give a final specific activity of 0.013 units/mg protein (32). However, several major protein contaminants were still present in the preparation at this stage of purity, suggesting that an alternative isolation protocol would be necessary to obtain enzyme with a higher specific activity. There are a number of protocols for the isolation of equine (33), rabbit (34), human (35), bovine (36) and porcine (37) plasma amine oxidase which could be used as a model for the enzyme from sheep plasma. A great deal of effort has been made to obtain pure preparations of bovine and porcine plasma amine oxidase with a high specific activity, and these were therefore chosen as a model for the sheep plasma enzyme. Porcine plasma amine oxidase with a specific activity of 0.105 units/mg protein when assayed with 3.33 mM benzylamine at 25°C has been obtained. Typically, the isolation of these plasma amine oxidase involves ammonium sulfate precipitation, anion-exchange chromatography, affinity chromatography on concanavalin-A lectin, gel filtration and chromatography on hydroxylapatite. The anion exchange and hydroxylapatite steps were useful in separating various isoforms of the porcine enzyme (37). In this work, a protocol based on the isolation of porcine plasma amine oxidase (37) was used to isolate a high specific activity fraction of sheep plasma amine oxidase and to separate four isoforms of the enzyme.

Materials and Methods

All chemicals were obtained commercially and were of reagent grade unless specified otherwise. Reverse osmosis water that was passed through anion exchange, cation exchange, carbon and 0.45 μ cartridge was used for buffers and chemical solutions.

Enzymes

Equine liver alcohol dehydrogenase, catalase, horseradish peroxidase, Type II, porcine kidney diamine oxidase and bovine plasma amine oxidase were obtained from Sigma. Porcine and bovine semicarbazide sensitive amine oxidases (SSAO) were isolated from aortic tissue of animals which were obtained from a local abattoir. Fresh porcine kidneys and clotted sheep blood were also obtained from local sources. All procedures were carried out at 4°C unless otherwise noted.

Activity and Protein Determination

All protein determinations were done using the Bio-Rad Kit (38) using bovine serum albumin as the standard protein. The activity of bovine and porcine SSAO at pH 7.6 was determined by incubation with 200 and 100 μM benzylamine, respectively, followed by quantitation of H_2O_2 using 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) as a chromogen and horseradish peroxidase (39). A standard curve was obtained by using a solution of hydrogen peroxide, standardized against a solution of potassium permanganate and an extinction coefficient of 23,000 $M^{-1}cm^{-1}$ at 414 nm for the oxidized 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid. Alternatively, production of benzaldehyde from the oxidation of benzylamine could be monitored directed at 252 nm, using an extinction coefficient of 11,500 $M^{-1}cm^{-1}$ for the aldehyde. Porcine kidney diamine oxidase activity was determined at pH 7.4 at 37°C using 3.3 mM putrescine as the substrate and 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) as a chromogen. Sheep plasma amine oxidase activity was monitored at pH 7.4 at 25°C using 3.3 mM benzylamine and following the production of benzaldehyde at 252 nm. A unit of activity was defined as the amount of enzyme which oxidized 1 μmole of substrate per minute at the temperature of the assay.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) was carried out using the Phast System (Pharmacia) or the Mini-Protean II apparatus (Bio-Rad). TEMED and ammonium persulfate were from Bio-Rad. Acrylamide and bisacrylamide were from American

Bioanalytical. Molecular weight protein standards were from Sigma. Chemicals used for buffers and sample preparation were of electrophoresis grade.

Denaturing, or sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the Phast system (Pharmacia) with 10 to 15% gradient polyacrylamide gels following manufacturer's instructions or with 1.5 mm, 11% polyacrylamide gels according to the discontinous system of Laemmli (40). For the Laemmli system, the stacking gel buffer was 0.12 M Tris-HCl, pH 6.8 with 0.1% sodium dodecylsulfate, the separating gel buffer was 0.38 M Tris-HCl, pH 8.8, with 0.1% sodium dodecylsulfate, and the running buffer was 20 mM Tris-glycine, pH 8.6, with 0.01% sodium dodecylsulfate. Samples were diluted in a 1:1 ratio with buffer containing 2% β-mercatoethanol, 1% sodium dodecyl sulfate, 0.1% bromophenol blue and 30% glycerol in 50 mM Tris-HCl, pH 6.8. SSAO samples were denatured by heating 3 minutes at 100°C and other proteins were denatured by heating 10 minutes at 85°C . Electrophoresis was carried out at 18 - 20 milliamperes until the marker dye, bromophenol blue, reached the bottom of the gel. A single step Coomassie blue staining procedure was used to visualize the protein bands (41).

Native-PAGE was performed according to a procedure for glycopeptides (42) with 1.5 mm and between 5 and 8% polyacrylamide gels. The stacking gel buffer was 0.12 M Tris-HCl, pH 6.8, the separating gel buffer was 0.25 M Tris-Borate, pH 8.3, the running buffer was 0.1 M Tris-Borate, pH 8.3, the sample buffer was 10 mM Tris-Borate, pH 8.3, with 0.1% bromophenol blue. The separation and protein detection was carried out as described above. Gels were cut in half, with one piece developed for protein and the other for activity. Activity of protein bands was determined using a coupled peroxidatic oxidation method, using tyramine as the substrate (43). For each gel, 2 mg of 3-amino-9-ethyl-carbazol was dissolved in 0.5 mL dimethyl formamide. To this, 7.5 mL of 30 mM sodium phosphate buffer, pH 7.6 was added and the solution was filtered through Whatman #2 filter paper. One mL of molten agar was added to the filtrate, along with 10 milligrams of horseradish peroxidase, Type II, and 1 mL of 33.3 mM tyramine. This solution was overlaid on the gel and incubated at 37°C for several hours or over night. Active bands appeared as lines of brown precipitate after the substrate mixture was washed away.

Solubilization and Isolation of Semicarbazide Sensitive Amine Oxidase

Excess fat and adherring tissue were removed from the aorta. After cleaning, the typical size of the bovine aorta was 160 grams, while the porcine aorta was 25 grams.

These were then rinsed in water, and put through a meat grinder (4 mm sieve). Ground tissue was then used immediately as an enzyme source or stored frozen at -30°C.

Enzyme was isolated from 200 to 500 gram batches of tissue. The following protocol is for a 200 gram sample of tissue. The tissue was washed in 1 L of 10 mM sodium phosphate, pH 7.6 by stirring for 15 minute and straining through 4 layers of cheesecloth. The buffer was discarded and the tissue blended using an Osterizer blender and 400 mL of the same buffer. The slurry was then stirred for 15 minutes and centrifuged at 10,000 x g for 30 minutes. The supernatant was discarded and the pellet resuspended in 400 mL 30 mM sodium phosphate, pH 7.6 with 1% Triton X-100. The bovine aortic tissue was extracted by stirring 2 hours and then centrifuged at 10,000 x g for 30 minutes. The supernatant was retained and the pellet was blended in 400 mL of the same buffer without detergent. Triton X-100 was then added to a final concentration of 1%, and the tissue was extracted a second time as above. Porcine aortic tissue was extracted with the same buffer as the bovine tissue but by stirring 6 hours for the first extraction and 18 hours for the second extraction.

After solubilization, the detergent extract was loaded at 2 mL/minute onto DEAE Sepharose CL6B column (5 x 20 cm) that was equilibrated with 30 mM sodium phosphate, pH 7.6. The column was washed with approximately 500 mL of the same buffer until the unbound protein had eluted, followed by 100 mM sodium phosphate, pH 7.6, 0.25 M NaCl. Activity was eluted in approximately 500 mL of this buffer . The enzyme was loaded at 1 mL/minute on 75 mL of a Sepharose 4B *Lens culinaris* lectin (2 mg lectin/mL gel) which was equilibrated with 0.1 mM of each $MgCl_2$, $CaCl_2$ and $MnCl_2$, and 0.5 M NaCl in 20 mM sodium phosphate, pH 7.6. The column was washed with equilibration buffer (200 mL) until protein levels were near background. A sharp peak of activity was eluted by loading a total volume of 200 mL of 1M α-methyl-D-glucose in sodium phosphate, pH 7.6 onto the column. After one column volume of this buffer had passed into the column, flow was halted and the buffer was allowed to remain in contact with the column for 4 hours or overnight. The active fractions eluted with the α-methyl-D-glucose-containing buffer were combined and concentrated using an Amicon ultrafiltration cell with a PM 30 membrane to less than 10 mL, and chromatographed on S-200 Sephacryl (2.5 x 95 cm) equilibrated with 20 mM sodium phosphate, pH 7.6, 0.1 M potassium chloride. A flow rate of 0.75 mL/min was used and 10 mL fractions were collected. Activity was eluted near 180 mL.

Isolation of Porcine Kidney Diamine Oxidase

Porcine kidney diamine oxidase was isolated from 3 kilograms of fresh pig cortices by modification of a previously described procedure (29). The cortices were homogenized in a Quisinart food processor in 2.5 liters of 30 mM sodium phosphate, pH 7.4. The slurry was centrifuged at 16,000 x g and the supernatant was filtered through cheesecloth. The 30 - 60% ammonium sulfate precipitate was obtained and redissolved in a minimum amount of 100 mM sodium phosphate, pH 7.4, for a total volume of approximately 400 mL. After dialysis against five changes of 1 L of 100 mM sodium phosphate, pH 7.4, the ammonium sulfate fraction was loaded at 2 mL/minute on a hydroxylapatite column (3.5 x 45 cm) equilibrated with the same buffer. Protein that was not retained on the column was removed by further washing with 100 mM sodium phosphate. Enzyme was eluted using a linear gradient of 400 mL each 0 and 1 M ammonium sulfate in the equilibration buffer. The active fractions were concentrated using an Amicon ultrafiltration cell with a PM 30 membrane to approximately 200 mL and dialyzed against two changes of three litres of 30 mM sodium phosphate buffer. The material was then loaded at 2 mL/minute onto a DEAE-Sephadex A-50 (4 x 45 cm) equilibrated with the same buffer. Activity was eluted by applying a linear gradient of 500 mL each 30 mM and 200 mM sodium phosphate buffer. With no further treatment, the enzyme was reapplied on a smaller hydroxylapatite column (2.5 x 20 cm) equilibrated with 100 mM sodium phosphate, pH 7.4, and activity was eluted as before with 200 mL of each 0 and 1 M ammonium sulfate in the equilibration buffer. The active fractions were concentrated by ultrafiltration to approximately 50 mL and dialyzed against 10 mM Tris-HCl, pH 7.4, 0.4 M NaCl. The dialyzed material was loaded at 1 mL/minute onto 30 mL of a Sepharose 4B *Lens culinaris* lectin (2 mg lectin/mL gel) which was equilibrated with the same buffer. Diamine oxidase was eluted with 1 M α-methyl-D-mannoside in 10 mM Tris-HCl, pH 7.4. Active fractions were concentrated by ultrafiltration to less than 10 mL and chromatographed on Sephacryl S-300 HR (2.5 x 100 cm) that was equilibrated with 100 mM sodium phosphate buffer. The flow rate was 0.5 mL/minute and 10 mL fractions were collected.

As well, smaller amounts of pig kidney diamine oxidase were isolated from a powder obtained from Sigma. The protocol used for the isolation of enzyme from this material was similar to that described above. In this case, hydroxylapatite chromatography was the first step, after dissolving and dialyzing the powder in 100 mM sodium phosphate, pH 7.4. As well, only a single hydroxylapatite step was used.

Isolation of Sheep Plasma Amine Oxidase

Sheep plasma amine oxidase was isolated using a protocol based on the isolation of porcine plasma amine oxidase (37). From 14 L of fresh, coagulated whole blood, 4.5 L of plasma was obtained. The plasma was centrifuged at 22,000 x g for 20 minutes and the 35 - 65% ammonium sulfate precipitate of the supernatant was obtained . The precipitate was dissolved in a minimum volume of 10 mM sodium phosphate buffer, pH 7.4, and the final volume of 750 mL was dialyzed 3 times against 12 L of the same buffer. Half of this material at a time was loaded at 2 mL/minute on DE-52 (5 x 20 cm) equilibrated with the same buffer. The majority of the activity was released from the column with approximately 700 mL of 30 mM sodium phosphate, pH 7.4 while a smaller amount was obtained using 300 mL of 100 mM phosphate, pH 7.4. This activity was loaded at 1 mL/minute onto Concanavalin A Sepharose lectin (2 mg lectin/mL gel) which was equilibrated with 1 mM of each $MgCl_2$, $CaCl_2$ and $MnCl_2$, and 150 mM NaCl in 20 mM sodium phosphate, pH 7.4. Enzyme was eluted with 20 mM sodium phosphate buffer, pH 7.4 with 150 mM α-methyl-D-mannoside and concentrated using an Amico ultrafiltration cell with a PM 30 membrane. This enzyme was subjected to gel filtration on Sephacryl S-200 HR equilibrated with 20 mM sodium phosphate, pH 7.5 containing 100 mM potassium chloride. The flow rate was 0.5 mL/minute and 18 mL fractions were collected. A fraction of the enzyme was rechromatographed as described above on a Concanavalin A column (2.5 x 10 cm) prepared by reductive amidation coupling (44), followed by gel filtration. This yielded an enzyme preparation with a specific activity of 0.13 - 0.15 units/ mg protein. The isoforms of the enzyme were separated by chromatography on an hydroxylapatite column (2.5 x 10 cm) equilibrated with 5 mM potassium phosphate, pH 7.4. Enzyme was eluted in both the equilibration buffer and 100 mM potassium phosphate, pH 7.4. Each peak was rechromatographed separately on a DE-52 column (2.5 x 10 cm) equilibrated with 10 mM potassium phosphate, pH 7.4. In both cases, a major band of activity was eluted with 30 mM potassium phosphate and a minor band eluted with the 100 mM potassium phosphate buffer.

Results and Discussion

Isolation and Partial Characterization of Bovine & Porcine Semicarbazide Sensitive Amine Oxidase

Semicarbazide sensitive amine oxidase was isolated from bovine and porcine aortic tissue (Tables 2.1 and 2.2) using a combination of procedures previously reported for rat brown adipose tissue (4) and cultured porcine aortic smooth muscle cells (17). During the isolation, 15% of enzyme was lost in the preliminary washing but this loss was tolerated since removal of possible contaminating plasma amine oxidase was necessary. Buffers containing 1 M sodium chloride had no solubilizing effect on the enzyme, indicating that SSAO may be an intrinsic membrane protein, or possibly a membrane anchored lipoprotein. As well, various wash buffers, including a borate buffer (45), a phosphate and a Tris buffer had no effect on the subsequent detergent extraction. The detergent CHAPS (3-(3-cholamidopropyl)dimethyl-ammonio-1-propane-sulfonate) was found to be effective in solubilizing the enzyme at 0.25 - 1% but 1% Triton X-100 was equally efficient and less expensive for the numbers of preparations that were required. The greatest amount of enzyme activity was obtained in the first detergent extraction. While a significant amount of activity was obtained in the second extraction, the yield was reduced to 30% of the first, and no further extractions were carried out. The amount of enzyme obtained from the tissue proved to be reproducible over a number of preparations that were carried out. The crude extracted enzyme was stable for several days at 4°C in the presence of Triton X-100, unlike monoamine oxidases which are unstable in the presence of this detergent (46). The disadvantage of using Triton X-100 is its low critical micelle concentration of 0.3 mM and large micelle size of 90,000 Daltons (47) which makes it difficult to remove from solutions by dialysis or ultrafiltration. As well, Triton X-100 is known to interfere with protein determinations by the Bradford method. Fortunately, this detergent was only required for initial solubilization of the enzyme, and inclusion of Triton X-100 at 0.1% in buffers used for subsequent steps did not influence yields or stability. Therefore, free detergent molecules or micelles could be virtually eliminated during the subsequent isolation steps. The lack of an absolute requirement for detergent is not typical of an intrinsic membrane protein (47), and it is likely that the protein binds detergent tightly at hydrophobic sites.

The enzyme showed variability in its behaviour during anion exchange chromatography. Some enzyme was eluted using only 100 mM phosphate buffer while a

Table 2.1. Purification of Bovine Semicarbazide Sensitive Amine Oxidase*

Step	Units	Specific Activity (Units/mg protein)	Yield (%)
Triton X-100 Extraction			
Extraction #1	17.0	0.0053	100
Extraction #2	5.3	0.0018	
DEAE-Sepharose CL6B	13.7	0.0081	48
Lentil lectin	3.0	0.062	13
S-200	1.6	0.11	7
	0.4	0.04	2

* 500 gm starting material; modelled on reference 4 and 17

Table 2.2. Purification of Porcine Semicarbazide Sensitive Amine Oxidase*

Step	Units	Specific Activity (Units/mg protein)	Yield (%)
Triton X-100 Extraction			
Extraction #1	2.5	0.00084	100
Extraction #2	0.7	0.00044	
DEAE-Sepharose CL6B	2.2	0.0015	69
Lentil lectin	0.5	0.0225	16
S-200	0.06	0.050	2
	0.27	0.027	8

* 475 gm starting material; modelled on reference 4 and 17

small amount of activity required harsher conditions of a decrease in pH to 6.6 and up to 1 M sodium chloride. Most activity was obtained with 100 mM phosphate buffer containing 0.25 M sodium chloride. As well, the solubilized enzyme required 2 to 3 sequential loadings onto the column, as some activity was found to flow through without adsorbing onto the column.

The affinity chromatography was the most effective step in increasing the specific activity of the enzyme. SSAO activity was found to be retained on both Concanavalin A and *Lens culinaris* lectins, establishing the glycoprotein nature of the enzyme. Low yields were obtained from Concanavalin A compared to *Lens culinaris*, indicating that the enzymes were highly glycosylated. While both lectins have an affinity for mannose and glucose moieties and their α-methyl derivatives, the binding constants for *Lens culinaris* lectin are approximately 50 fold lower than for Concanavalin A (48). SSAO could be eluted from the *Lens culinaris* lectin using a 1M solution of either α-methyl-D-mannose or α-methyl-D-glucose. Enzyme was also eluted at lower concentrations of sugar, but yields were lower and a larger volume was required. This may be an effect of variations in the levels of glycosylation of the enzymes, which causes a range of affinities for the lectin. Such heterogeneity was suggested to be present in porcine plasma (37) and bovine plasma amine oxidases (49).

The lectin affinity step was also important for the removal of lysyl oxidase from SSAO. Aortic tissue has high levels of lysyl oxidase which are released with 4 M urea buffers. The requirement for such a harsh treatment for solubilization is a strong indication that the enzyme is associated with a cellular membrane (50). It might be expected therefore, that some lysyl oxidase might be solubilized in the Triton X-100 detergent. Lysyl oxidase from bovine aorta, however, has been shown to have no affinity for Con-A Sepharose (51) and is therefore eliminated with this step.

The overall yield of the isolations from each source was less than 10% with a 20 to 60 fold increase in specific activity. The yield is lower than that obtained from either rat brown adipose tissue (4) or culture porcine aortic smooth muscle cells (17). However, a slightly higher specific activity was obtained with this protocol, which combined the methodologies of these previously published works. Activity from the cultured porcine aortic smooth muscle cells was reported to be purified to homogeneity as determined by SDS-PAGE, while in this work, several proteins were observed on gels (see discussion below). This observation, and the comparable specific activities that were obtained by the two methods suggests that less enzyme inactivation occurred in the protocol developed in this work. Alternatively, the enzyme from the cultured cells may have a lower turnover

number than that isolated from the aortic tissue. The enzymes retained full activity for several months at 4°C. The final preparations of porcine and bovine SSAO were pale yellow with a featureless spectrum. This is in contrast to the plasma amine oxidases which are descibed as being peach or pink, a characteristic attributed to the cofactor of these enzymes, 2,4,5-trihydroxyphenylalanine (TOPA) (23).

Polyacrylamide gels of both the bovine and porcine SSAO showed that several major bands were present in the final preparations of the enzymes (Figure 2.1). Previous reports indicated that the enzyme from rat brown adipose tissue was a dimer composed of two monomers of approximately 90,000 Daltons (4). Therefore, the band in each gel with a molecular weight near 90,000 Daltons is tentatively attributed to SSAO. From the gels, the molecular weight of the bovine enzyme is estimated to be 92,000 while the porcine enzyme is estimated as 95,000. These values may be an overestimation of the molecular weight of the subunits of SSAO. Glycoproteins have been reported to exhibit anomalous behaviour on SDS-PAGE if they contain charged carbohydrates such as sialic acid or sulfates (52). These charged groups reduce the binding of SDS from a uniform 1.4 gm/gm protein found with other proteins, resulting in lower mobility than expected, based on their true molecular size. However, Barrand and Callingham found the subunit molecular weight of rat brown adipose tissue SSAO by SDS-PAGE to correspond well to the molecular weight of the membrane bound active dimer when radiation inactivation was used as an independent method to evaluate molecular size (4). Since this enzyme and the aortic enzymes behaved similarly on the lectin columns, their levels and type of glycosylation appear to be similar, and the molecular weight by SDS-PAGE of the aortic enzymes is believed to be accurate.

Both enzymes display a high affinity for benzylamine with a Km value for bovine SSAO of 16 μM (± 2) and for the porcine SSAO of 8 μM (± 1). Estimates of Km were obtained using a weighted least squares linear regression program (53). These values are similar to those for SSAO from several rat sources (4,18) and from cultured porcine aortic cells (17). The Km values are significantly lower than those for the plasma enzyme of each source which is 1.7 mM for bovine plasma (54) and 90 μM for porcine plasma (55) amine oxidase. Bovine and porcine SSAO displayed substrate inhibition at benzylamine concentrations greater than 200 and 100 μM, respectively. Substrate inhibition has also been noted for copper containing amine oxidases (56). The significance of this effect with benzylamine is not clear, as it is an unnatural substrate for the enzyme but, in general, substrate inhibition may be a method of controlling the activity of SSAO *in vivo*. There are no reports of substrate inhibition with methylamine, another good substrate for SSAO

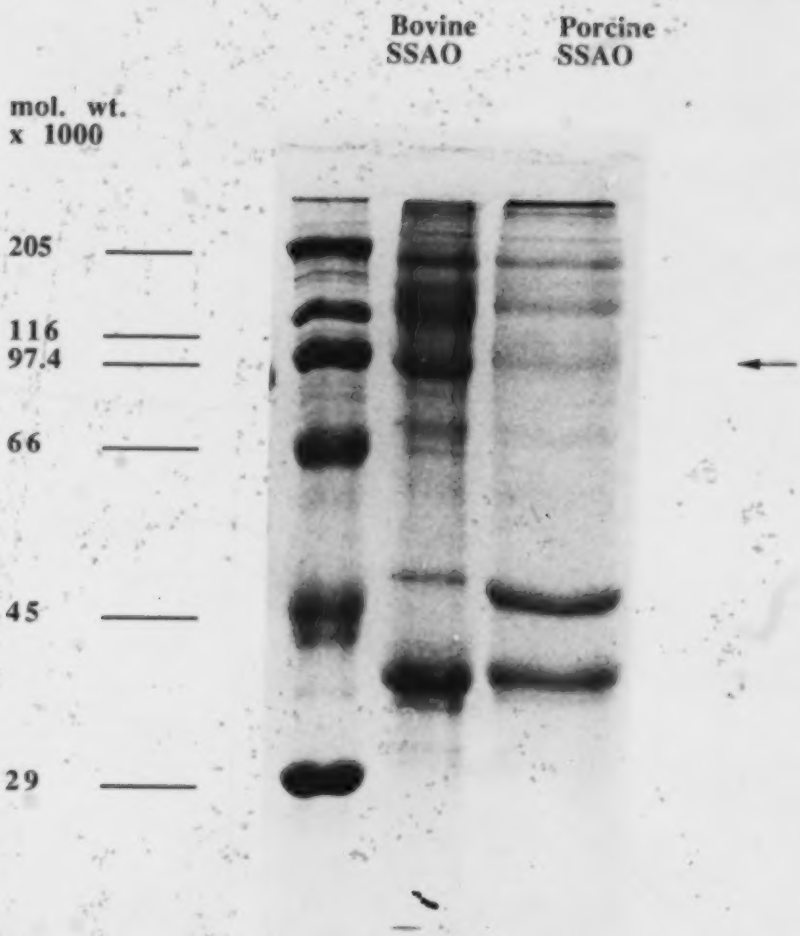


Figure 2.1 SDS-PAGE of bovine and porcine semicarbazide sensitive amine oxidase

enzymes but with a low Km value for this substrate of 102 μM (57), such physiological control may be feasible.

A comparison of the relative activity of bovine and porcine SSAO with a variety of amines is given in Table 2.3 and shows that the substrate profile for both enzymes was very similar. The enzymes had very low diamine oxidase activity at 200 μM concentration of cadaverine and putrescine. Histamine and tryptamine were poor substrates while tyramine, β-phenethylamine and methylamine were better, although still oxidized at a reduced rate compared to benzylamine. This pattern is similar to that seen for other SSAO (6).

Table 2.3. Relative Activity of Bovine and Porcine Semicarbazide Sensitive Amine Oxidase with a Panel of Amines*

Amine	% Relative Activity	
	Bovine	Porcine
Benzylamine	100	100
Methylamine	81	127
β-Phenethylamine	49	79
Tyramine	34	73
Tryptamine	10	20
Histamine	9	17
Putrescine	4	11
Cadaverine	3	10

* 200 μM concentration, pH 7.6

The irreversible inhibition of porcine and bovine SSAO by semicarbazide was also demonstrated (Table 2.4). An aliquot of enzyme was reacted with the inhibitor at a final concentration of 10 μM and 100 μM. The solutions were kept at 4°C for 24 hours, excess inhibitor was removed using a PD-10 column (Pharmacia) and the enzyme was concentrated using a Centricon 10 unit (Amicon) or dialysis tubing (12,000-14,000 molecular weight cut off) coated with dextrans (266,000 molecular weight). For comparison, the inhibition of bovine plasma amine oxidase was also examined. At 10 μM, the bovine plasma amine oxidase was inhibited more quickly than the SSAO. The plasma enzyme appeared to be fully inactivated after 2 hours at this concentration, while a similar level of inhibition was reached within 24 hours for the SSAO. This effect was less

obvious with the 100 μM concentration of semicarbazide. The variation in the susceptibility of the plasma amine oxidase and aortic amine oxidase to semicarbazide may indicate that the cofactor in these enzymes is not equally accessible. The inhibition of SSAO and plasma amine oxidase was irreversible as determined by a lack of reactivation after removal of excess semicarbazide, and suggests that the cofactor contains a carbonyl moiety.

Table 2.4. Inhibition of Amine Oxidases with Semicarbazide

Semicarbazide (μM)	Percent Inhibition of Enzyme					
	Bovine SSAO		Porcine SSAO		Bovine Plasma	
	2 hrs	24 hrs	2 hrs	24 hrs	2hrs	24 hrs
0	0	0	0	0	0	0
10	27	78	37	78	90	94
100	93	96	94	100	97	97

Phenylhydrazine is also a carbonyl reagent and an irreversible inhibitor of copper containing amine oxidases (58). The reactivity of phenylhydrazine with these enzymes provides the basis for a spectral test for the presence of the cofactor, 2,4,5-trihydroxyphenylalanine, TOPA (23). TOPA has been shown to form a phenylhydrazone adduct under neutral pH conditions with an absorbance maximum near 450 nm. After removal of excess phenylhydrazine and addition of KOH to a final concentration greater than 2 M, the absorbance peak undergoes a significant red shift to near 485 nm. Bovine plasma amine oxidases, which has been shown to contain TOPA by independent methods, display these chromophores. Although bovine and porcine SSAO were irreversibly inhibited by 1 mM phenylhydrazine, the adducts formed were spectrally distinct from those formed with the plasma amine oxidases. At pH 7.6, a pale yellow chromophore with an absorbance maximum at 428 nm was formed with bovine SSAO (Figure 2.2). With multiple additions of 10 M potassium hydroxide to a final concentration of 2.8 M, a decrease in this absorbance maximum was seen, with no other changes noted in the spectrum. This data suggests that the cofactor in the SSAO enzymes is a carbonyl type molecule but that it is not TOPA.

There are several physio-chemical properties which suggest that aortic SSAO is unique from other copper containing amine oxidases. The higher affinity for benzylamine exhibited by the SSAO enzymes is unique from other benzylamine oxidizing enzymes such

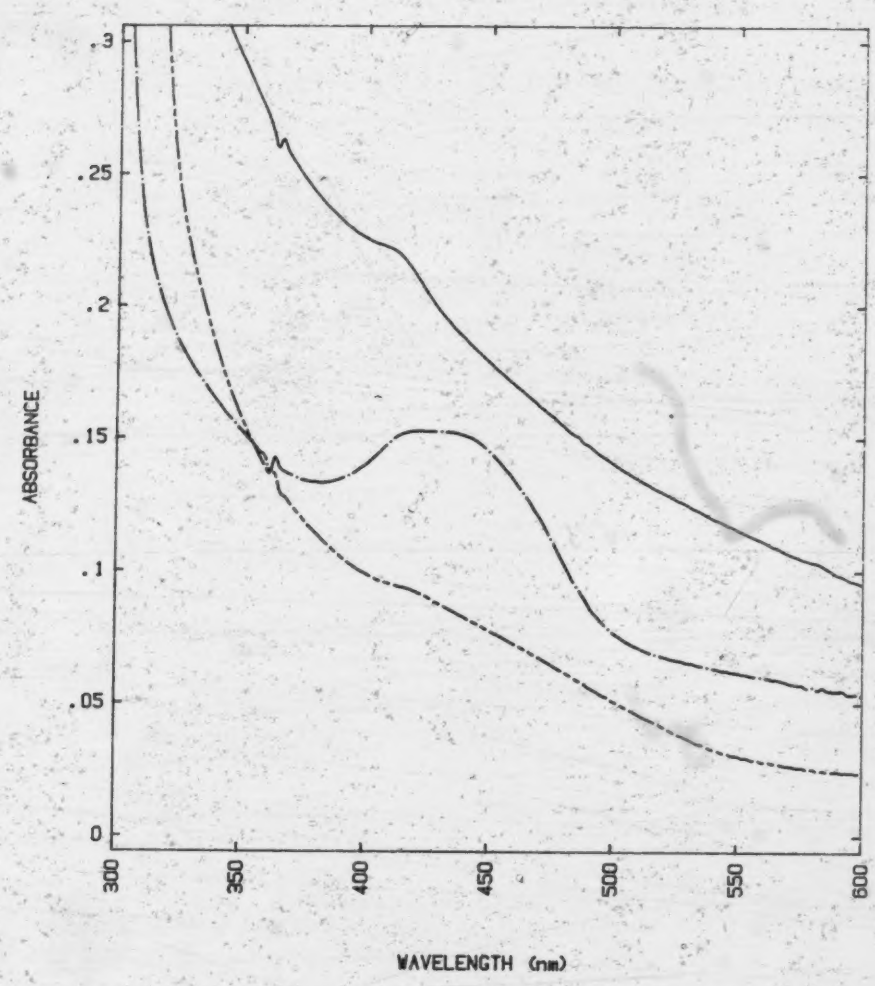


Figure 2.2. Spectra of bovine semicarbazide sensitive amine oxidase
(a) native enzyme (———)
(1.3 mg protein/ mL, final conc.)
(b) with 1 mM phenylhydrazine (·———·)
(0.9 mg protein/mL, final conc.)
(c) as in (b) with 2.8 M KOH (-———)
(0.8 mg/mL, final conc.)

as those from the plasma. The spectral properties of SSAO are distinct and they do not appear to contain the chromophore giving the peach or pink colour associated with the copper containing enzymes. As well, SSAO reactivity with semicarbazide and phenylhydrazine differs from that of copper containing amine oxidases. While the nature of the cofactor remains an unknown, evidence suggests that it is not pyridoxal phosphate, but a quinone type molecule. However, the SSAO enzymes gave a negative response in the test for the presence of TOPA . Future work in the laboratory will be directed towards the elucidation of the cofactor structure.

Isolation of Porcine Kidney Diamine Oxidase

The porcine kidney diamine oxidase was obtained from fresh kidney cortices with a final specific activity of 1.1 units/mg protein (Table 2.5). Estimations of the yields of the preparation were based on the activity that was found to be present after the ammonium sulfate precipitation step. The initial estimate of the enzyme activity in the homogenate was inaccurate, and may be the related to the nature of the assay used. Activity was determined

Table 2.5. Purification of Porcine Kidney Diamine Oxidase*

Step	Units	Specific Activity (Units/mg protein)	Yield (%)
Supernatant	96	0.0016	---
30 - 60% $(NH_4)_2SO_4$	143	0.0030	100
Hydroxylapatite	139	0.038	97
DE-52	105	0.082	74
Hydroxylapatite	123	0.14	84
Lentil lectin	65	0.74	46
S-300	67	1.1	47

* isolated from 3 kg porcine kidney cortices; modified from reference 29

using 2,2' azino-di(3-ethylbenzthiazoline-6-sulfonic acid) as a chromogen, and relies on the detection of hydrogen peroxide as one of the products of the amine oxidase reaction. Some of the hydrogen peroxide may have been destroyed by endogenous catalase in the homogenate, causing an underestimation of the initial activity present.

A heat treatment was not used in this protocol, even though it is often included in preparations (25, 27). Reports of yields and specific activities with this step varied considerably, and are likely related to efficiency of both heating and cooling of the enzyme solution. As well, protocols without this step have been developed which match or improve on the purity and yield obtained with procedures including the heat treatment (28). The final preparation had an orange hue, with an absorption maximum at 414 nm. This peak may be due to an impurity in the preparation as previous reports of the absorbance maximum of the enzyme place the peak at 480 nm (59). An SDS-PAGE of the enzyme showed one major band with a molecular weight of approximately 97,000 (Figure 2.3). The enzyme is reported to have a monomer molecular weight of 91,000 by SDS-PAGE (60).

The effect of carbonyl reagents on pig kidney diamine oxidase have been examined in the past. Semicarbazide and phenylhydrazine completely inhibited the enzyme at 1 μM concentration (27), and a red shift in the absorbance peak of the phenylhydrazine adduct occurred under basic conditions, suggesting the presence of TOPA (23).

Isolation of Sheep Plasma Amine Oxidase

Previously, the sheep plasma amine oxidase had been isolated to 0.013 units/mg protein (32). The protocol used in our work resulted in a more highly purified enzyme with a specific activity of 0.071 units/mg protein after a single pass through the lectin and gel filtration columns (Table 2.6). Fractions with a specific activity of 0.13 to 0.15 units/mg protein were obtained by successive rechromatographing the enzyme on a concanavalin A column prepared by reductive amidation and an S-200 gel filtration column.

The enzyme had a pale pink colour, typical of the plasma amine oxidases, and the absorption spectrum had a small shoulder near 480 nm.

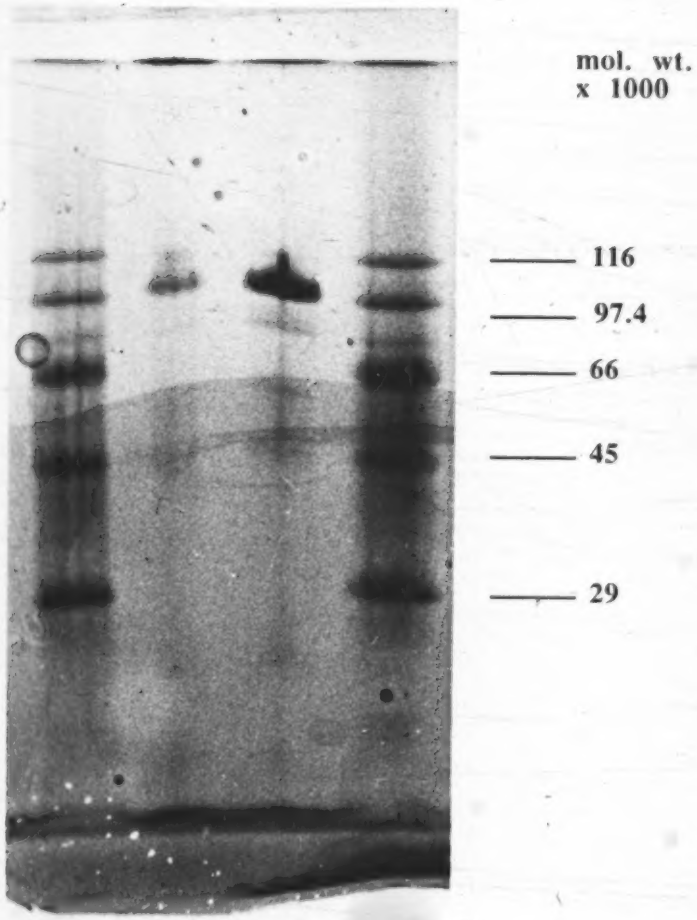


Figure 2.3 SDS-PAGE of porcine kidney diamine oxidase

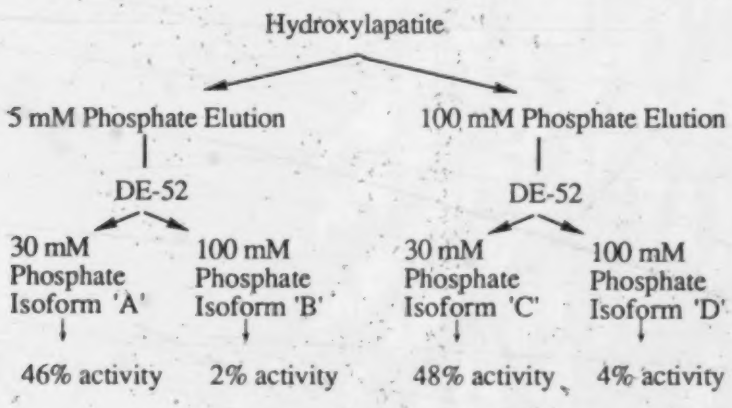
Table 2.6. Purification of Sheep Plasma Amine Oxidase*

Step	Units	Specific Activity (Units/mg protein)	Yield (%)
Serum	90	0.00036	100
35 - 65% $(NH_4)_2SO_4$	79	0.00091	88
DE-52			
30 mM	50	0.0020	55
100 mM	8	0.0007	9
Con A-Sepharose	34	0.020	38
S-200	20	0.071	22

* isolated from 14 L fresh blood; modified from reference 37

The enzymes behaviour during the isolation was similar to that of porcine plasma enzyme and four isoforms of the sheep plasma amine oxidase were separated by sequential chromatography on a hydroxylapatite and anion exchange column (Figure 2.4). Isoforms of the porcine plasma amine oxidase have also been isolated in a similar manner (37).

Figure 2.4. Separation of Sheep Plasma Amine Oxidase Isoforms



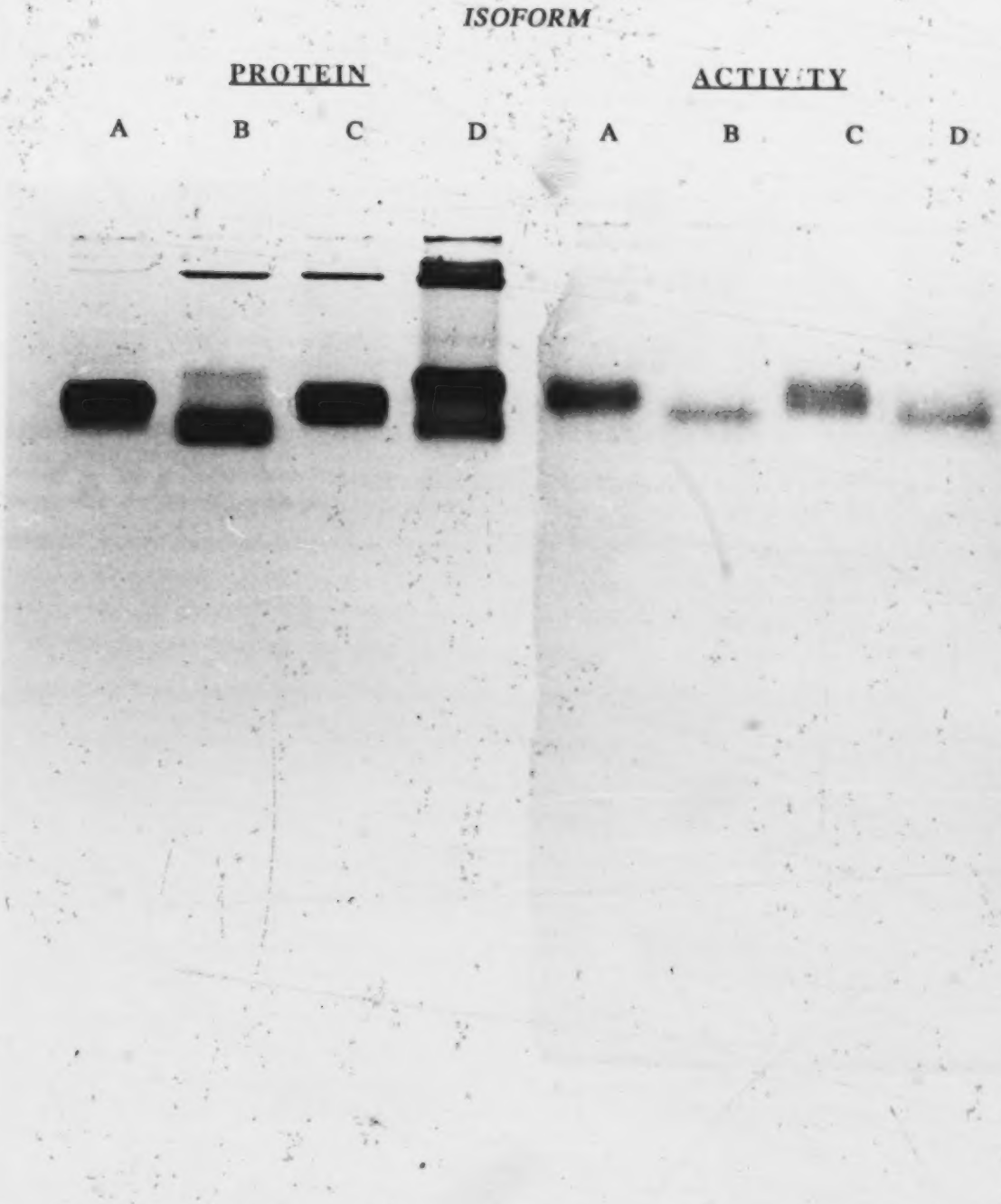


Figure 2.5. Native-PAGE of sheep plasma amine oxidase

However, differences in the distribution of the isoforms of the enzymes were apparent. Both sources of the enzyme were partioned almost equally between activity eluted with a high and low ionic strength buffer from hydroxylapatite. However, the porcine plasma amine oxidase fractions obtained with a low ionic buffer from anion exchange chromatography accounted for 32% of the total activity, while the major sheep plasma amine oxidase isoforms, accounting for 94% of the activity of the preparation, were obtained under the same conditions. The four isoforms of the sheep enzyme showed slight migration differences during non-denaturing polyacrylamide gel electrophoresis (Figure 2.5). The Tris-borate buffer system used in the native-PAGE influences the migration of glycoproteins in the gel. Under basic conditions, charged borate ester complexes may be formed with neutral sugars. The number of borate ions bound to a glycoprotein then, is dependent on the composition, linkages and amounts of carbohydrates present. The migration differences seen may be due, in part, to variations in glycosylation on the enzyme. A similar level of variation in glycosylation has been noted for bovine (49) and porcine plasma amine oxidase (37). As well, since the isoforms of the enzymes displayed charge variations during anion exchange and hydroxylapatite chromatography, migration difference during electrophoresis might be expected.

The presence of TOPA as the cofactor of the sheep plasma amine oxidase was suggested, based on the spectral characterization of the phenylhydrazine adduct. Upon addition of 1 mM phenylhydrazine to the enzyme, a peak with an absorbance maximum at 444 nm was immediately formed. After removing the free phenylhydrazine, and addition of potassium hydroxide to 4.8 M, a red shift in the absorbance maximum to near 480 nm was seen (Figure 2.6). Similar spectra were obtained with bovine plasma amine oxidase, which has been shown to contain TOPA by independent methods (23).

Sufficient amounts of the SSAO enzymes from bovine and porcine aortic tissue and the copper amine oxidases from porcine kidney and sheep plasma were isolated to carry out the stereochemical studies, described in Chapters 3 and 4.

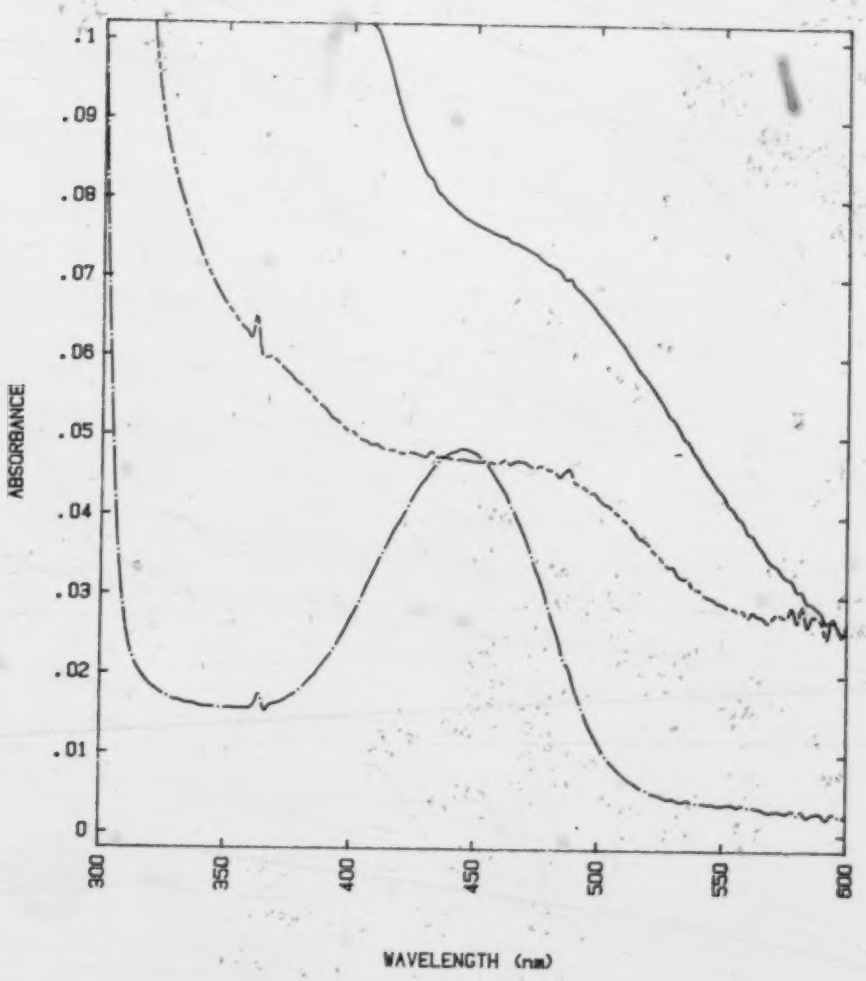


Figure 2.6. Spectra of sheep plasma amine oxidase
(a) native enzyme (———)
(0.4 mg protein/mL, final conc.)
(b) with 1 mM phenylhydrazine (-·-·-·-)
(0.3 mg protein/mL, final conc.)
(c) as in (b) with 4.8 M KOH (- - - - -)
(0.2 mg protein/mL, final conc.)

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Chapter 3. Stereochemical Course of Tyramine Oxidation by Semicarbazide Sensitive and Copper Amine Oxidase

Introduction

The cofactor of semicarbazide sensitive amine oxidase was partially characterized and several physio-chemical properties of this enzyme were investigated in Chapter 2. These studies suggested that semicarbazide sensitive amine oxidases (SSAO) constitute a distinct class that is unique from the copper and flavin containing amine oxidases. Usually, the basis for classification in the Enzyme Commission (E.C.) system is the chemical reaction catalyzed by an enzyme. Consequently, each name and E.C. number designates not a single protein but rather a group of proteins catalyzing the same reaction. However, separate entries are made in instances where the reaction mechanism is different as observed for the flavin monoamine oxidases and the copper amine oxidases which contain different cofactors. By the same token, a separate E.C. number may be deemed appropriate for the SSAO enzymes.

To acquire further information on the relationship between the SSAO and copper amine oxidases, sufficient amounts of SSAO were prepared to carry out stereochemical studies. Hence, the stereochemical course of tyramine oxidation catalyzed by the porcine and bovine aorta SSAO enzymes and the copper amine oxidases from sheep plasma and porcine kidney has been elucidated and is reported in this chapter.

Both the SSAO and the copper amine oxidases catalyze the oxidative deamination of amines to aldehydes with the release of hydrogen peroxide and ammonia. With the copper amine oxidases, this is proposed to occur via an imine intermediate (1,2), consistent with a transaminase mechanism (3) (Equation 3.1).

$$E_{ox} + RCH_2NH_3^+ \rightleftharpoons E_{red}{=}NH^+CH_2R \rightleftharpoons E_{red}\text{-}NH^+{=}CHR + H_2O \quad (3.1)$$
$$\rightarrow E_{red}NH_2 + O{=}CH\text{-}CH_2\text{-}R + O_2 \rightarrow E_{ox} + H_2O_2 + NH_4^+$$

After the formation of an imine intermediate between cofactor and substrate, a proton is abstracted from C-1 of the substrate in what is suggested to be a base catalyzed reaction (4). There are three possible modes for this abstraction - pro-*R*, pro-*S* or an apparent nonstereospecific abstraction[1]. Through the introduction of different hydrogen isotopes,

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[1] The stereochemical terminology of pro-*S* (or pro-*R*) refers to the proton of a pro-chiral methylene which if given higher priority in the Cahn-Ingold-Prelog system, generates a chiral center with an *S* (or *R*) configuration.

the prochiral methylene centre of aliphatic and aromatic amines may be made chiral, and the stereochemical course of the C-1 proton abstraction during the reaction can be determined. In examining the stereochemistry of the copper amine oxidases, use has been made of both deuterium and tritium labeled amines.

Investigations into the stereochemistry of the copper amine oxidase reaction has thus far uncovered a surprising heterogeneity, as all three possible modes of C-1 proton abstraction have been observed within this one class of enzyme (5), as typified by the following examples. The stereochemical abstraction mode for porcine kidney diamine oxidase was first established as pro-*S* with histamine (6), and this was confirmed with other substrates including cadaverine, putrescine and histamine (7). Alternatively, the porcine plasma enzyme was found to abstract the pro-*R* proton from C-1 of dopamine and tyramine (5), while the bovine plasma amine oxidase showed an apparent nonstereospecificity with dopamine, β-phenethylamine and tyramine (4, 5). While nonstereospecificity in a reaction is generally taken as a strong indication that it is not enzyme-mediated, this result was attributed to the existence of two catalytically competent substrate binding modes in the enzyme active site (4).

Such stereochemical diversity within one formal classification of enzymes has not been observed with any other group of enzymes. The mechanistic basis for this nonuniformity is not known and is highly unusual since the stereochemistry of related enzymes tends to be conserved to maintain the overall chemistry and efficiency of the transformation (8). For example, the FAD-dependent monoamine oxidases, which catalyze the same type of reaction as the copper containing and semicarbazide sensitive enzymes, have been found to oxidize amines with the abstraction of the pro-*R* proton at C-1. This is true of FAD monoamine oxidases that have been isolated from a variety of tissues and different animal species (9) and with aliphatic and aromatic substrates (10).

There is evidence that the stereochemistry of the reaction at C-1 for some copper amine oxidases is substrate dependent. For example, in contrast to the nonstereospecificity of bovine plasma amine oxidase with dopamine and β-phenethylamine described above, this enzyme catalyzes the abstraction of the pro-*S* proton of benzylamine (11), *p*-hydroxybenzylamine (1) and 3-methylbutylamine (12). As well, more subtle differences in mechanism related to substrate conformation have been suggested. For example, porcine kidney diamine oxidase exhibits an isotope effect on the proton abstraction at C-1 during the oxidation of putrescine but not with cadaverine (7). Based on our current understanding of these enzymes, then, only a limited amount of confidence can be placed in a prediction of the stereochemical course of a reaction of an enzyme from a new source or with a 'new' substrate.

In addition to the main catalytic pathway of amine oxidases, a side reaction of imine-enamine tautomerization has been found to occur in some copper containing enzymes prior to imine hydrolysis (Equation 3.2).

$$E_{red}\text{-}NH^{+}\text{=}CH\text{-}CH_2\text{-}R \rightleftharpoons E_{red}\text{-}NH\text{-}CH\text{=}CH\text{-}R + H^{+} \qquad (3.2)$$

In this pathway, a proton at the β, or C-2, position of the substrate may be exchanged with solvent. The existence of this side reaction was first reported for bovine plasma amine oxidase, which was shown to catalyze the release of 50% of the C-2 [^{3}H] from dopamine (13). However, the unavailability of chiral amines of known configuration precluded determination of the stereochemistry of this reaction. As well, porcine kidney diamine oxidase was found to deaminate histamine with loss of tritium from the β-position but tritium was not released from the β-position of putrescine (14). This suggests that expression of the imine-enamine tautomerization pathway is both enzyme and substrate dependent, as is the stereochemistry at C-1.

A variety of techniques have been used to determine the stereochemical course of amine oxidase reactions. All have required the use of stereospecifically labelled amines of known configuration. One of the earliest methods of determining the stereochemistry of an amine oxidase reaction involved calculating the kinetic isotope effects of rat liver monoamine oxidase using deuterium labelled tyramine (15). However, there are limitations to the general applicability of this methodology, since proton abstraction may not be a rate limiting step during catalysis. Such is the case with the porcine kidney diamine oxidase which, as described above, has an isotope effect on the proton abstraction with putrescine but not one with cadaverine (7).

Dual labelling of substrates with ^{3}H and ^{14}C has also been used. A comparison of the ratios of the two isotopes in substrate and products was used to determine the stereochemistry of pea seedling amine oxidase which was found to catalyze the release of the pro-*S* proton at C-1 (16). However, this is an indirect method of determining stereochemistry, as is the use of kinetic isotope effects.

It is preferrable to use a methodology that allows the direct monitoring of products of the reactions. This can be accomplished by chromatographic separation of products, or spectroscopic methods such as nuclear magnetic resonance (NMR) or mass spectroscopy. Reverse phase HPLC has been used to differentiate protio- and monodeutro-aldehydes of several amine oxidase reactions, although resolution of the two species was incomplete (17). As well, mass spectroscopy has been used to detect products of benzylamine

oxidation by amine oxidases (18). Both proton (5) and deuterium (7) nuclear magnetic resonance have been used to determine the stereochemistry of amine oxidase reactions.

The present study has made use of ^{1}H-NMR and chirally deuterated tyramines to determine the stereochemical course of oxidation by semicarbazide sensitive amine oxidase, and to compare this to the stereochemistry of two copper containing amine oxidases, porcine kidney diamine oxidase and sheep plasma amine oxidase. The stereochemistry of bovine and porcine aortic semicarbazide sensitive amine oxidase have not been previously examined. Information concerning the relationship of these SSAO to other copper amine oxidases can therefore be established from the stereochemical patterns exhibited by the enzymes. The stereochemistry of porcine kidney diamine oxidase was previously established as pro-*S* with several substrates, but the reaction with tyramine has not been examined. As well, it is not clear if the solvent exchange reaction should occur with this substrate. The sheep plasma amine oxidase reaction was examined in earlier work with a preparation containing several major contaminating proteins and found to catalyze an apparent nonstereospecific reaction with tyramine. To establish that this behaviour is intrinsic to the plasma enzyme, and not due to the presence of alternate amine oxidases, the stereochemistry of a high specific activity sheep plasma amine oxidase was determined. As well, the stereochemistry of the reaction catalyzed by two major isoforms of the sheep plasma enzyme were examined.

Materials and Methods

All chemicals were of reagent grade unless otherwise specified and all protonated solvents were distilled before use. Deuterated solvents were obtained from Merck, Sharp and Dohme (99.95% ^{2}H), or General Intermediates of Canada (99.9% ^{2}H). Reverse osmosis water that was passed through an anion exchange, cation exchange, carbon and 0.45 μm cartridges was used for all chemical solutions and buffers. The (1R)-[^{2}H] and (1S)-[^{2}H] tyramines were available from previous studies and their synthesis is given in Appendix I.

Enzymes

Horse liver alcohol dehydrogenase, catalase and tyrosine decarboxylase from *Streptococcus faecalis* were obtained from Sigma. Semicarbazide sensitive amine oxidase from bovine and porcine aortic tissue, sheep plasma amine oxidase and porcine kidney amine oxidase were isolated as outlined in Chapter 2.

Stereochemical Course of Tyramine Oxidation at C-1

The enzymatic oxidation of amines was coupled to alcohol production with alcohol dehydrogenase (16). To this end, 0.3 units of amine oxidase, were incubated in 3 to 5 mL of 30 mM phosphate buffer, pH 7.6 containing 10 µmol of (1*R*)- or (1*S*)- [^{2}H]tyramine, 25 µmol of NADH, 4 units of alcohol dehydrogenase and 11,000 units of catalase. Incubations were carried out at room temperature (23°C) or 37°C. The reactions were allowed to proceed until 50% of the reaction had been completed, as determined by a decrease in absorbance at 340 nm caused by the oxidation of NADH, or overnight. After completion of the reaction, samples were loaded onto C-18 Sep Pak cartridges (Waters), the cartridges were washed with 5 mL water and then alcohol products were eluted with 10 mL methanol. The methanol eluate was concentrated to dryness under reduced pressure and chromatographed on a 2 gm flash silica column (60-100 mesh, BDH) using ethyl acetate: hexane (2:1) as a solvent. After drying, samples were used for ^{1}H-NMR analysis. Alternatively, enzyme incubations were extracted 3 times with 25 mL of ethyl acetate instead of applying to Sep Pak cartridges. Successive extracts were evaporated to dryness under reduced pressure, chromatographed on a flash silica column and analyzed by ^{1}H-NMR as described above.

^{1}H-NMR Spectroscopy

^{1}H-NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument at ambient temperature (22 + 1°C). The spectra of *p*-hydroxyphenethyl alcohols were obtained in $(^2H_3C)_2CO$:^2H_2O (4:1), with the residual H^2H_2C signal set at 2.19 as an internal reference standard. Optimization of the conditions for obtaining NMR spectra was determined by Dr. O. Hindsgaul[2]. The spectra of tyramine hydrochlorides were obtained in ^2H_2O using the residual H^2HO signal at 4.81 as an internal reference standard. Under these conditions, absolute chemical shifts were found to be reproducible to less than 0.05 ppm. Spectra were accumulated into 16 K of computer memory using a 45° pulse with a 7 second relaxation delay between pulses. Relaxation was complete, as longer delays did not alter the relative intensities of the signals as determined by integration. The spectra of protonated tyramine, C-1-deuterated chiral tyramines, and fully protonated *p*-hydroxyphenethyl alcohol were used to obtain the chemical shifts of the proton signals.

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[2] Department of Chemistry, University of Alberta

The method used to calculate the amount of monoprotonated and diprotonated product alcohols from the ^{1}H-NMR spectra is given in Appendix II.

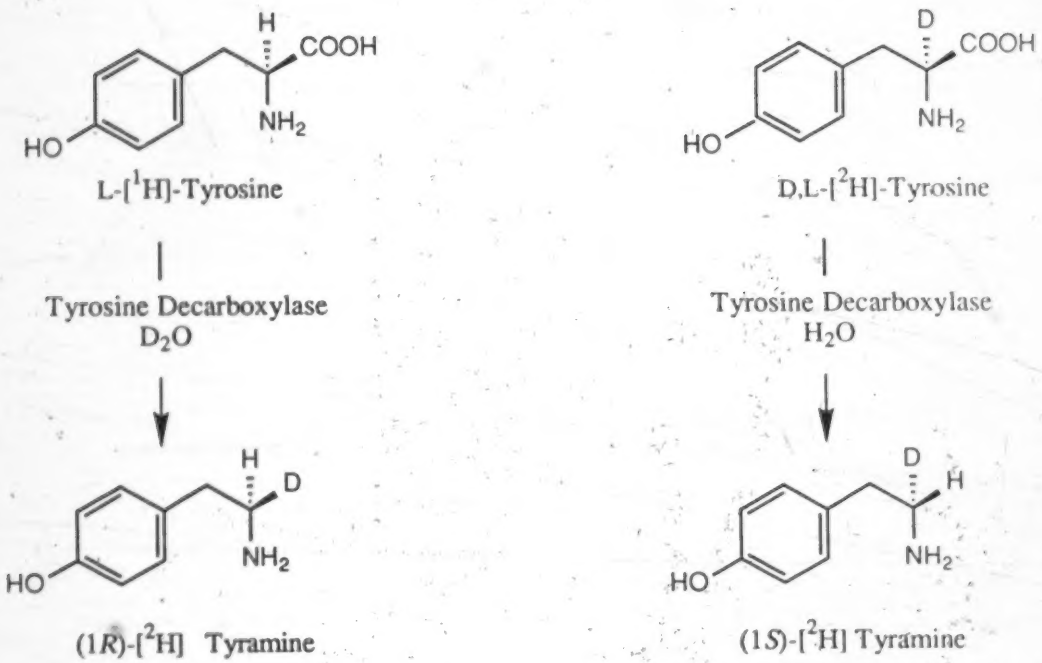
Solvent Exchange into p-Hydroxyphenethyl Alcohols

Incubations to determine solvent exchange into alcohols were carried out under similar conditions to those used for determining the stereochemistry of tyramine oxidation at C-1 with the following changes. Fully protonate tyramine was used as the substrate and reactions were carried out in 20 mM deuterated potassium phosphate buffer, pD 6.9. Deuterated potassium phosphate buffer was obtained by evaporating 100 mL of 0.2 M protonated buffer, pH 7.2, to dryness under reduced pressure. The buffer salts were redissolved in 20 mL ^2H_2O and dried, two times, and finally reconstituted in 20 mL of ^2H_2O. Enzyme was also exchanged into deuterated buffer by addition of 10 mL of buffer to 1 mL of enzyme and concentrating in an Amicon ultrafiltration cell with a PM-30 membrane to 1 mL. This was repeated 3 times. Product alcohols were isolated and ^{1}H-NMR spectra were obtained as described above.

Results and Discussion

In this work, chiral C-1 deuterated tyramines were obtained enzymatically by the decarboxylation of C-1 protonated and deuterated tyrosine with tyrosine decarboxylase from *Streptococcus faecalis* (Appendix I). Since the reaction of this enzyme with tyrosine is known to proceed with greater than 95% retention of configuration (19), parallel incubations can be used to obtain both (1*R*)-[^{2}H] and (1*S*)-[^{2}H] amines (Scheme 3.1). The ^{1}H-NMR spectra of both (1*S*)-[^{2}H] and (1*R*)-[^{2}H] tyramines obtained in this manner are shown in Figure 3.1 and confirm that deuterium incorporation at the C-1 position of each amine is nearly complete. The signal attributed to the C-1 proton is the triplet near 3.2 ppm, while the signal for the C-2 protons is a doublet near 2.9 ppm. Previous stereochemical studies using these same amines confirmed their configurational purity as being greater than 95% (5).

In the stereochemical studies, amines were converted to aldehyde products by the amine oxidases. The aldehydes were then coupled directly to alcohol production using alcohol dehydrogenase and NADH to eliminate nonenzymatic oxidation and Schiff base formation (Scheme 3.2). The deuterium content of the *p*-hydroxyphenyl alcohol products compared to the substrates served as a marker for the steric course of the reaction. Retention of deuterium in product alcohols resulted in an ^{1}H-NMR spectrum consisting of a triplet attributed to the C-1 proton signals and a doublet for the C-2 proton signals, similar



Scheme 3.1. Enzymatic synthesis of (1*S*)-[^{2}H] and (1*R*)-[^{2}H] tyramine

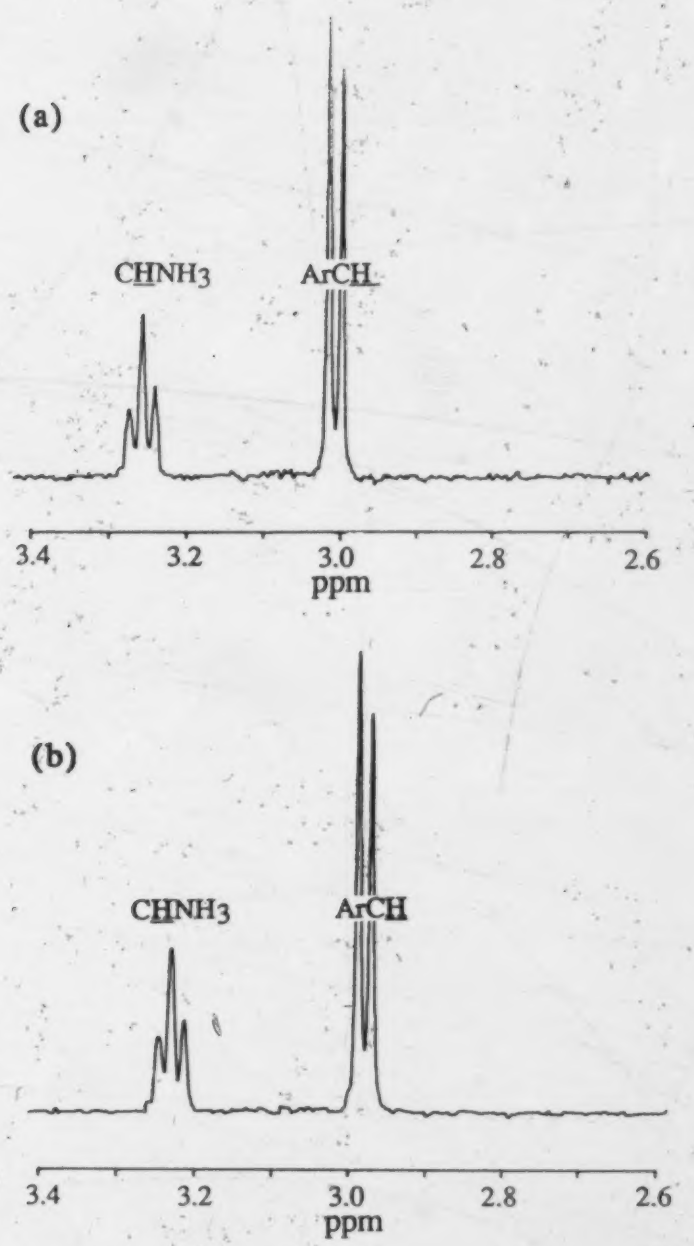
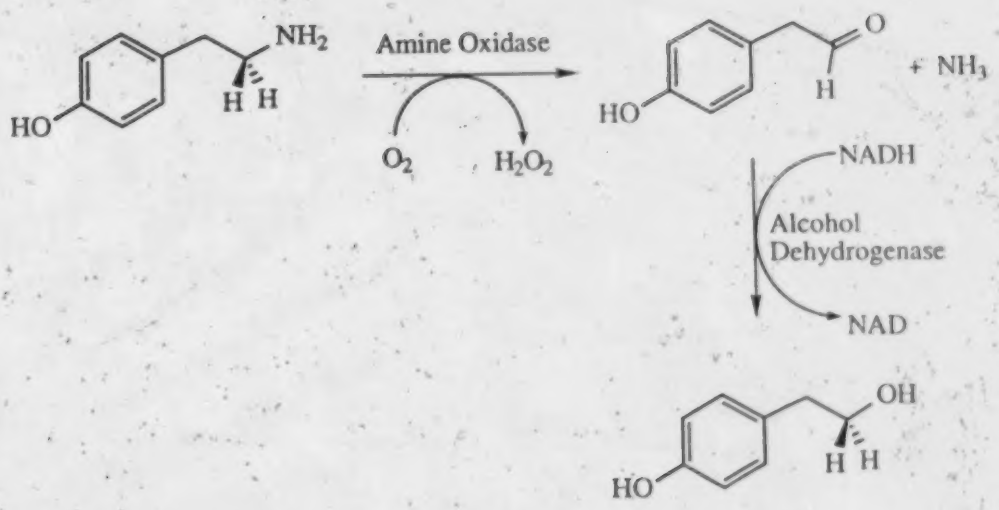


Figure 3.1. ^{1}H-NMR spectra of (a) (1*R*)-[^{2}H] tyramine and (b) (1*S*)-[^{2}H] tyramine derived from incubations of tyrosine decarboxylase



Scheme 3.2 Amine oxidase reaction coupled to alcohol production via alcohol dehydrogenase

to the original amine but with chemical shifts near 3.8 ppm and 2.7 ppm, respectively. A loss of deuterium resulted in an alcohol with a signal of a triplet for both C-1 and C-2 protons, with shifts near 3.8 ppm and 2.7 ppm, respectively. As will be shown, a distinct pattern of tyramine oxidation and solvent exchange was obtained for the SSAO, the sheep plasma and the porcine kidney amine oxidases. Of particular interest is the pattern of tyramine oxidation and solvent exchange observed for the bovine and porcine SSAO that was unique from that seen with any copper amine oxidase.

Bovine and Porcine Semicarbazide Sensitive Amine Oxidase

Tyramine oxidation proceeded with the cleavage of the pro-*S* proton by both the bovine and porcine semicarbazide sensitive amine oxidases. This is evident, for each enzyme, from the alcohol products obtained from incubations with (1*R*)-[^{2}H] tyramine. The spectra of the products exhibited mainly a triplet near 3.8 ppm and a doublet near 2.7 ppm, indicating retention of the majority of the deuterium (Figure 3.2). However minor signals corresponding to two triplets from fully protonated product are also present at approximately the same ppm, indicating that a small amount of deuterium had been lost in the reaction. The major signals near 3.7 and 2.8 ppm are shifted 0.02 ppm upfield due to an α- and β-deuterium isotope effect, respectively (20). The spectra from the corresponding alcohol products from the (1*S*)-[^{2}H] tyramine incubation are composed mainly of a triplet near 3.8 ppm and a triplet near 2.7 ppm , indicating that the majority of the deuterium had been lost from the substrate (Figure 3.3). Again, the stereochemistry was not absolute, as a small amount of monodeuterated product was isolated from the reaction. This minor product was more difficult to detect, since the doublet near 2.7 ppm is completely hidden under the triplet and only integration of the individual peaks of the triplet indicates a deviation from the expected 1:3:1 ratio. However, the triplet near 3.8 ppm can be seen as it is shifted slightly upfield from the larger triplet of the major product.

Duplicate incubations of (1*R*)-[^{2}H] tyramine with bovine semicarbazide sensitive amine oxidase resulted in 7 and 11% diprotonated product, with the residual 93 and 89% of the product being monodeuterated. When (1*S*)-[^{2}H] tyramine was used as the starting material, 91 and 89% of the product was diprotonated, and 9 and 11% of the product was monodeuterated. Similarly, 13 and 11% of the product was diprotonate when (1*R*)-[^{2}H] tyramine were used as substrate for the porcine semicarbazide sensitive amine oxidase while 90 and 86% of the product was diprotonate when (1*S*)-[^{2}H] tyramine were used as substrate. The retention and loss of deuterium in the products from the *S*- and *R*- modes is complementary for both enzymes, and the presence of an isotope in the scissile bond did not affect the stereochemical mode of the enzyme. This behaviour suggests a lack of an

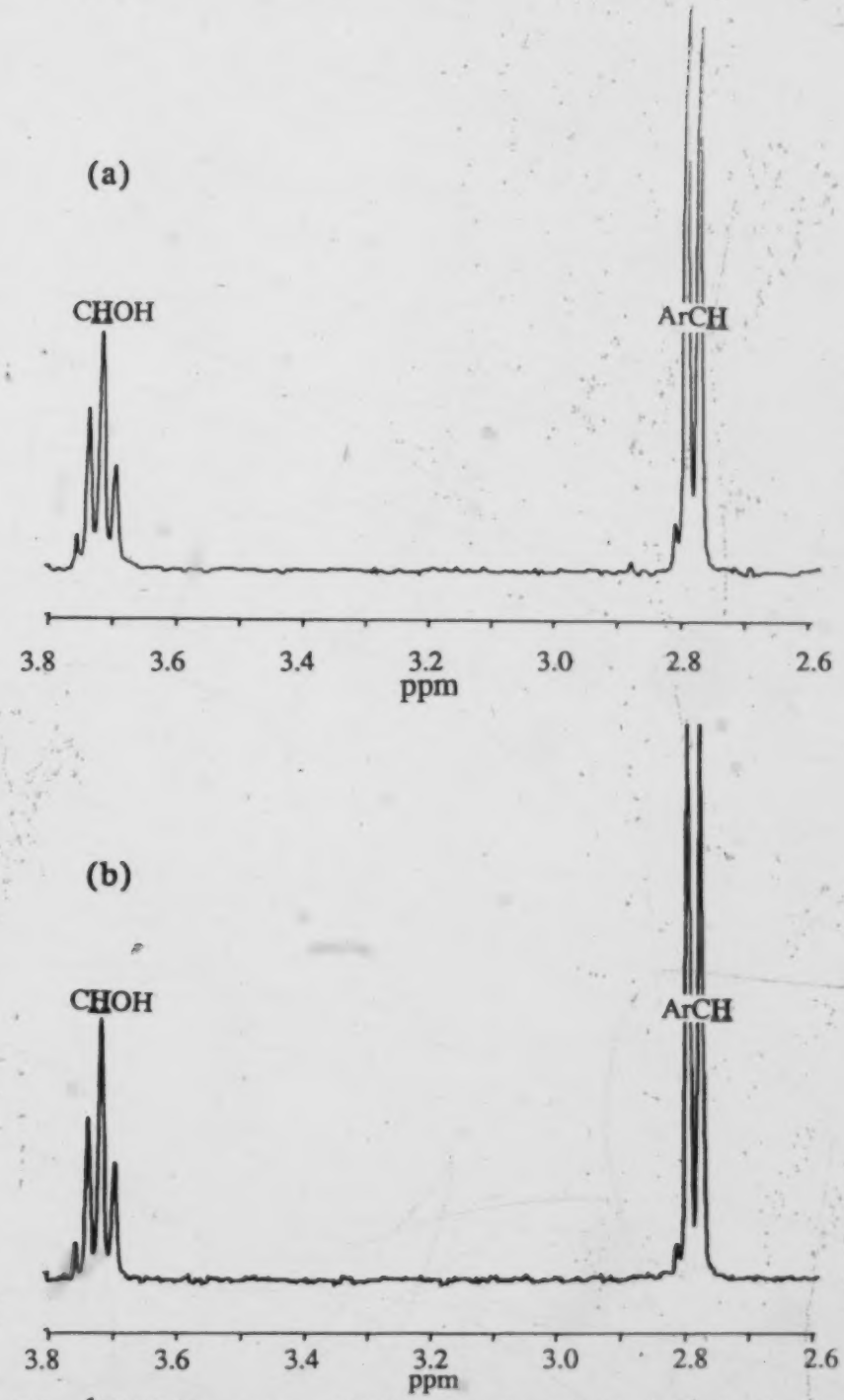


Figure 3.2. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols derived from coupled incubations with (1*R*)-[^{2}H] tyramine by (a) bovine SSAO and (b) porcine SSAO

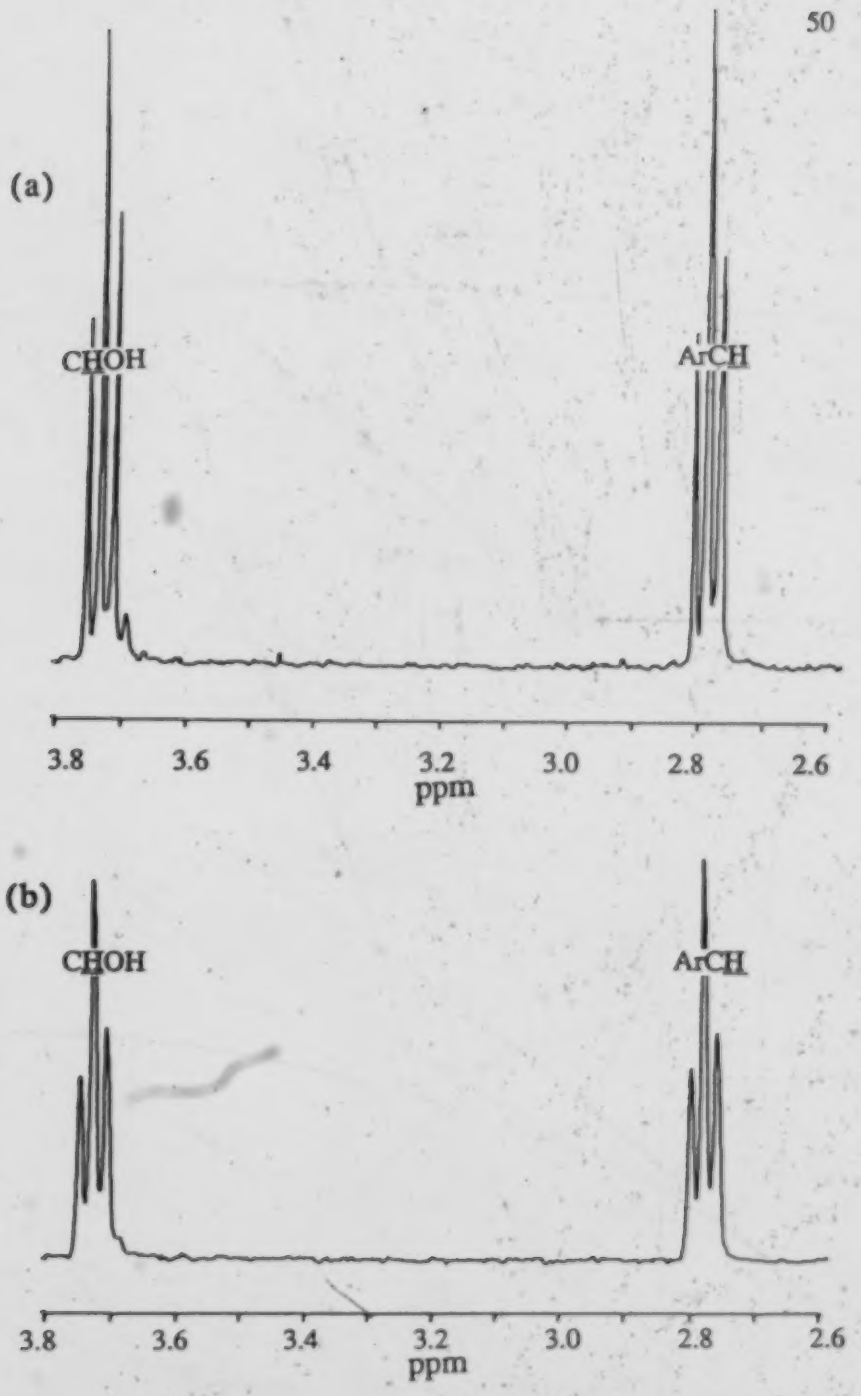


Figure 3.3. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols derived from coupled incubations with (1*S*)-[^{2}H] tyramine by (a) bovine SSAO and (b) porcine SSAO

isotope effect on proton abstraction at C-1 of tyramine by the SSAO enzymes, which remains to be confirmed by determining the presence or absence of kinetic isotope effects with protonated and chirally deuterated substrates. The expression of an isotope effect on C-1 proton abstraction has been shown to be substrate dependent for some copper amine oxidases. For example, the porcine kidney diamine oxidase displays an isotope effect on proton abstraction with putrescine but not with cadaverine (7).

These results suggest that approximately 5% of the reaction of both the bovine and porcine SSAO occurred by pro-*R* proton abstraction, since the starting chiral amine substrates contained about 5% diprotonated material (Figure 3.1). This limited amount of pro-*R* proton abstraction may be due to the presence of a second activity, but this seems unlikely since similar results were obtained with two independent preparations at differing levels of purity. Although incomplete coupling of aldehyde to alcohol production could be responsible for some scrambling of deuterium at C-1, this is unlikely since at least a 10-fold concentration of alcohol dehydrogenase and a 3-fold excess of NADH was used.

There is precedence for a lack of stereospecificity in the copper amine oxidase reaction. A lack of absolute stereochemistry was noted with the oxidation of (*R*)- and (*S*)-3[1-^{2}H] methylbutylamines by the bovine plasma enzyme (12). In this case, the enzyme was found to catalyze the abstraction of mainly the pro-*S* proton but a considerable amount of pro-*R* proton was also removed. With the *S*-deuterated substrate, deuterium was lost from 66% of the product and retained in 34%. The product from the *R*-deuterated substrate exhibited close to the opposite ratio, with deuterium being lost from 32% and retained in 68%. However, the stereochemical course of the reaction of bovine plasma amine oxidase was not influenced by the presence of an isotope and the ratios of monodeuterated and diprotonated products obtained from the *R*- and *S*- deuterated substrates were complementary.

The predominantly pro-*S* proton abstraction at C-1 catalyzed by the SSAO, coupled with the insensitivity of the reaction to the position of the isotope suggests that the SSAOs exhibit a single binding mode for tyramine. This binding optimally positions C-1 for efficient abstraction of the pro-*S* proton while the pro-*R* proton is in a more hindered position. This scenario has been put forward to explain the stereochemical behaviour of serine hydroxymethyltransferase and tryptophan synthase (21). These enzymes have been shown to lack absolute stereospecificity for the α-protons of glycine, but to exhibit a marked preference for one stereochemical mode over the other. Serine hydroxymethyltransferase was found to abstract the pro-*S* proton 7400 times faster than the pro-*R* proton while tryptophan synthase preferentially catalyzed the exchange of the pro-*R* protons of glycine 380 times faster than the pro-*S* protons. It was proposed that the

these enzymes exhibited a single binding mode for substrate which allowed an active site base unhindered access to one proton and only limited access to the second proton.

Alternatively, the substrate may be able to bind in two productive modes, such that either of the C-1 protons of tyramine may be abstracted, and the ratio of pro-*R* to pro-*S* abstraction would depend on the populations of the two modes. This rare phenomenon of dual binding modes for substrates has been suggested for bovine plasma amine oxidase to account for the apparent nonstereospecific behaviour of this enzyme with dopamine (4) and tyramine (5). In the case of the bovine plasma amine oxidase, the mode of the reaction is strongly influenced by the presence of the isotope while we have shown that the mode of the SSAO reaction was virtually independent of the presence of an isotope. For the SSAO enzymes then, it is probable that a single binding mode exists for tyramine and that the ratio of proton abstraction from the pro-*S* and pro-*R* modes is dependent on active site conformation.

Typically, the copper amine oxidases examined to date, display a propensity for pro-*S* proton abstraction over the alternate mode, as was observed for the SSAO. This is exhibited as simply pro-*S* proton abstraction as with the pea seedling amine oxidase (16), or in the case of nonstereospecificity of bovine plasma amine oxidase, as a more fully expressed isotope effect for pro-*S* proton abstraction compared to the pro-*R* mode (4). An interpretation of the bovine plasma amine oxidase nonstereospecificity has been put forward by Farnham and Klinman (22) who suggested that the configuration of the active site of bovine plasma amine oxidase accomodated the pro-*S* mode more easily than the pro-*R* mode. In the latter case, a partially rate limiting conformational change was required after the initial enzyme-substrate interaction before catalysis could proceed, reducing the full expression of the isotope effect. Conversely, C-H bond cleavage in the pro-*S* mode did not require a configurational modification, and therefore the C-H bond cleavage was more fully rate limiting. The only exception to the predominance of the pro-*S* proton abstraction in the copper containing amine oxidases is the porcine plasma amine oxidase which exhibits strict pro-*R* proton abstraction with dopamine and tyramine (5).

The stereochemistry of another SSAO has been reported. Rat aorta SSAO oxidation of dopamine (17) and benzylamine (18) was found to proceed with loss of the pro-*S* proton. Therefore, the SSAO enzymes from aorta appear to exhibit a consistent stereochemistry between animal species and for substrates with and without a β-methylene group. However, these reports of the rat aorta SSAO stereochemistry gave no indication that a small amount of pro-*R* proton was also abstracted, as was observed in this work. The separation and detection of protonated and monodeuterated aldehyde products of the rat enzyme was obtained by reverse phase HPLC with electrochemical detection. However,

resolution between product species was incomplete, and the small amount of pro-*R* proton abstraction may not have been detected.

The kinetic isotope effects calculated for the rat aortic SSAO also suggested that a limited amount of pro-*R* cleavage occurred as was found in our work on bovine and porcine amine oxidase. The pro-*R* C-1 proton cleavage was partially rate limiting in the mechanism of rat aortic semicarbazide sensitive amine oxidase (17). Kinetic isotope effects for this enzyme were calculated for both the pro-*S* and pro-*R* proton abstraction of dopamine and benzylamine. For dopamine, an isotope effect of 1.3 for V_H/V_D and 1.2 for $(V/K)_H/(V/K)_D$ was noted for pro-*R* proton cleavage, compared to 4.3 for V_H/V_D and 5.8 for $(V/K)_H/(V/K)_D$ for pro-*S* proton cleavage. The lower isotope effect for pro-*R* proton cleavage was attributed by the author to a secondary isotope effect. In view of the small amount of flux through the pro-*R* pathway found in this work, it is possible that this is a reflection of a small primary isotope effect. The similarity in values for the isotope effect on V_H/V_D and $(V/K)_H/(V/K)_D$ for dopamine oxidation suggests that enzyme reoxidation and product release are not rate limiting steps in the mechanism of this enzyme.

Both the bovine and porcine SSAO catalyze the exchange of protons at C-2 of tyramine with solvent. The *p*-hydroxyphenethyl alcohol isolated from the solvent exchange incubations for each enzyme exhibited a doublet signal for C-1 near 3.7 ppm and a triplet for C-2 near 2.8 ppm, indicating that deuterium had been incorporated into the C-2 position (Figure 3.4). Integrations of product NMR spectra from duplicate incubations of the bovine SSAO resulted in 0.97 and 1.04 deuterium atoms incorporated at C-2. However, a small amount (<5%) of diprotonated product was present in both incubations and visible, since the signals from the major monodeuterated product are shifted approximately 0.02 ppm upfield. This indicates that the error associated with integration of NMR signals was ≤ 5%. Nearly identical results were obtained from duplicate incubations for the porcine SSAO, as 0.97 and 1.04 deuterium atoms were incorporated at C-2 of product, and a small amount of diprotonated product was visible in the spectra. The nearly complete solvent exchange at C-2 of products suggests that the enamine pathway competes well with the main hydrolytic pathway. This may occur if an active site base is correctly positioned and present in the active unionized state, ready for proton abstraction. However, from this work it is not possible to determine if proton abstraction at C-1 and C-2 is base catalyzed, and if one or two bases are involved. Further detail regarding the stereochemistry of the imine-enamine tautomerization reaction, as well as pH dependency of catalysis would aid in elucidating this information. The stereochemistry of the solvent exchange reaction will be presented in Chapter 4.

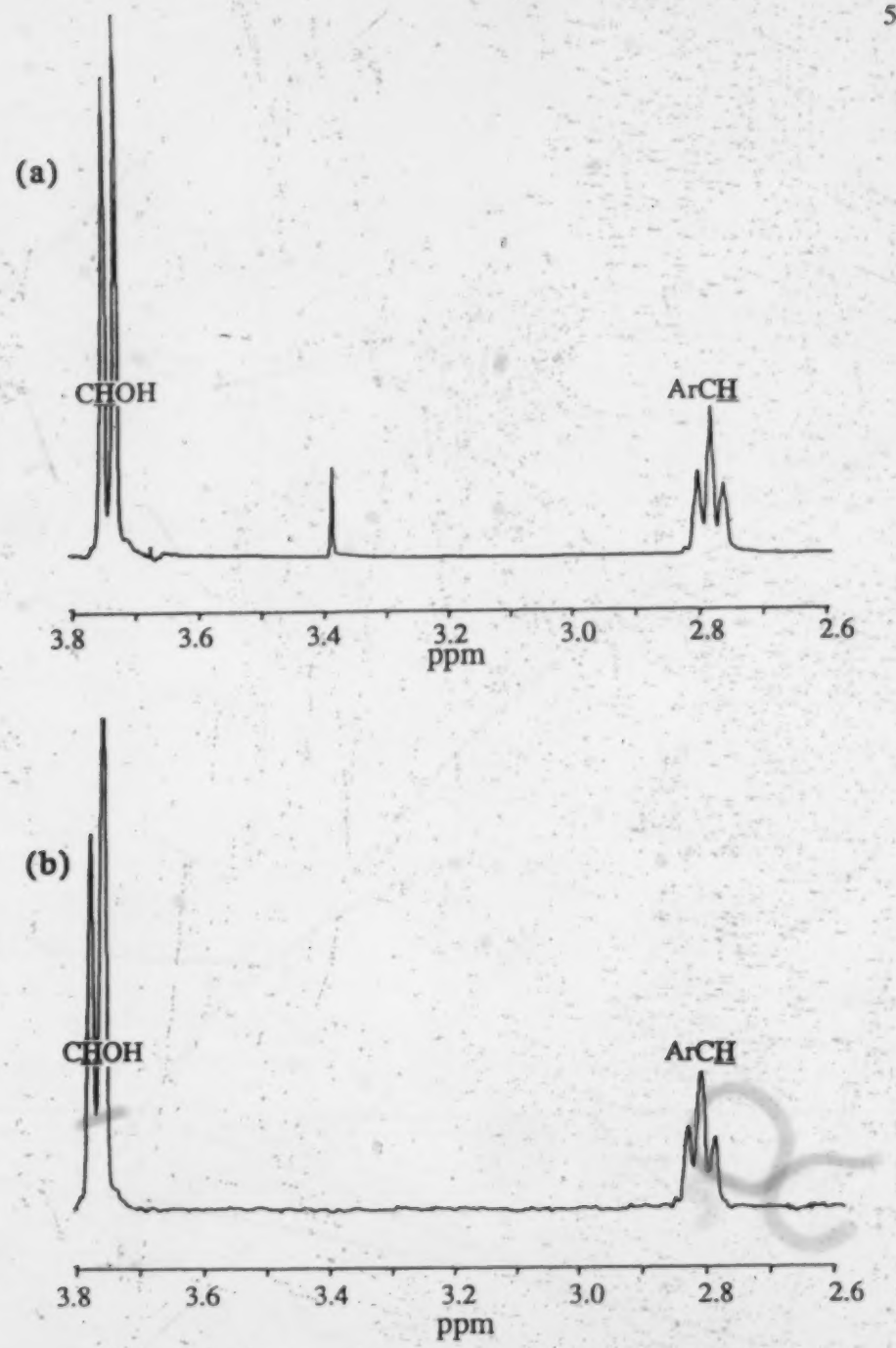


Figure 3.4. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols derived from coupled incubations with [1,1-^{1}H] tyramine in $^{2}H_2O$ by (a) bovine SSAO and (b) porcine SSAO

The solvent exchange reaction, together with the pro-*S* stereochemistry at C-1, distinguish the SSAO enzymes from the copper amine oxidases. To date, the copper amine oxidases which have exhibited pro-*S* proton abstraction at C-1 of tyramine, have not catalyzed the imine-enamine tautomerization reaction with tyramine (Table 3.1). This includes pea, chick pea and soybean seedling enzymes (23), and the porcine kidney diamine oxidase, as will be discussed below. As well, the copper amine oxidases which do catalyze the C-2 proton exchange, exhibit alternate stereochemistries at C-1 and incorporate different amounts of deuterium than the SSAO enzymes. For example, 0.8 or 1.1 deuterium atoms were incorporated by bovine plasma amine oxidase while 1.2 were incorporated by the enzyme from porcine plasma (5). The configuration of the active site residues of SSAO enzymes may be altered from that of the copper amine oxidase enzymes, and this in turn may affect the efficiency with which solvent exchange competes with the main catalytic pathway.

Porcine Kidney Diamine Oxidase

Porcine kidney diamine oxidase was found to oxidize tyramine with abstraction of the pro-*S* hydrogen as expected. This is evident from the ^{1}H-NMR spectra of the *p*-hydroxyphenethyl alcohols isolated from the incubations with (1*R*)-[^{2}H] and (1*S*)-[^{2}H] tyramines (Figure 3.5). The spectrum of the alcohol from the (1*R*)-[^{2}H] tyramine contains a doublet near 2.8 ppm and a triplet near 3.7 ppm, indicating that the deuterium label had been retained. Approximately 8% of the diprotonate product was also formed in this reaction. The corresponding incubation with (1*S*)-[^{2}H] tyramine exhibited two triplets with the same chemical shifts, as would be expected from a fully protonated compound with no evidence of monodeuterated product being formed. It is unclear why some scrambling occurred with the loss of deuterium with only the (1*R*)-[^{2}H] tyramine. However, it is evident that the position of the isotope does not affect the stereochemical mode of the reaction, which proceeds with the pro-*S* specificity. This is the first account of the stereochemistry of the porcine kidney diamine oxidase with tyramine as a substrate, although the reaction with a variety of other substrates including histidine (6), cadaverine, putrescine, agmatine (7), dopamine (17) and benzylamine (18) have been examined in the past. In each of these cases, porcine kidney diamine oxidase reaction proceeded with pro-*S* stereochemistry as was observed in this work.

There is evidence, however, that the mechanism of porcine kidney diamine oxidase varies with substrate, as C-1 proton cleavage was rate limiting for putrescine but not for a closely related substrate, cadaverine (7). It is of interest to note that these two substrates differ in molecular structure by a single methylene group. The stereochemistry of the

Table 3.1 Stereochemistry at C-1 of tyramine and solvent exchange at C-2 catalyzed by semicarbazide sensitive and copper amine oxidases

Enzyme Source	C-1 Proton Abstraction	C-2 Solvent Exchange	# Deuteriums Incorporated
Semicarbazide Sensitive Amine Oxidases			
Porcine Aorta[a]	pro-S	Yes	0.97, 1.04
Bovine Aorta[a]	pro-S	Yes	0.97, 1.04
Copper Amine Oxidases (E.C. 1.4.3.6)			
Porcine Plasma[b]	pro-R	Yes	1.20
Bovine Plasma[b,c]	nonstereospecific	Yes	1.10, 0.80
Sheep Plasma[a,d]	nonstereospecific	Yes	0.94
Rabbit Plasma[d]	nonstereospecific	Yes	0.74
Porcine Kidney[a]	pro-S	No	
Pea Seedling[b,e]	pro-S	No	
Soybean Seedling[d]	pro-S	No	
Chick Pea Seedling[d]	pro-S	No	

a this work
b Ref. 5
c Ref. 4
d Ref. 23
e Ref. 16

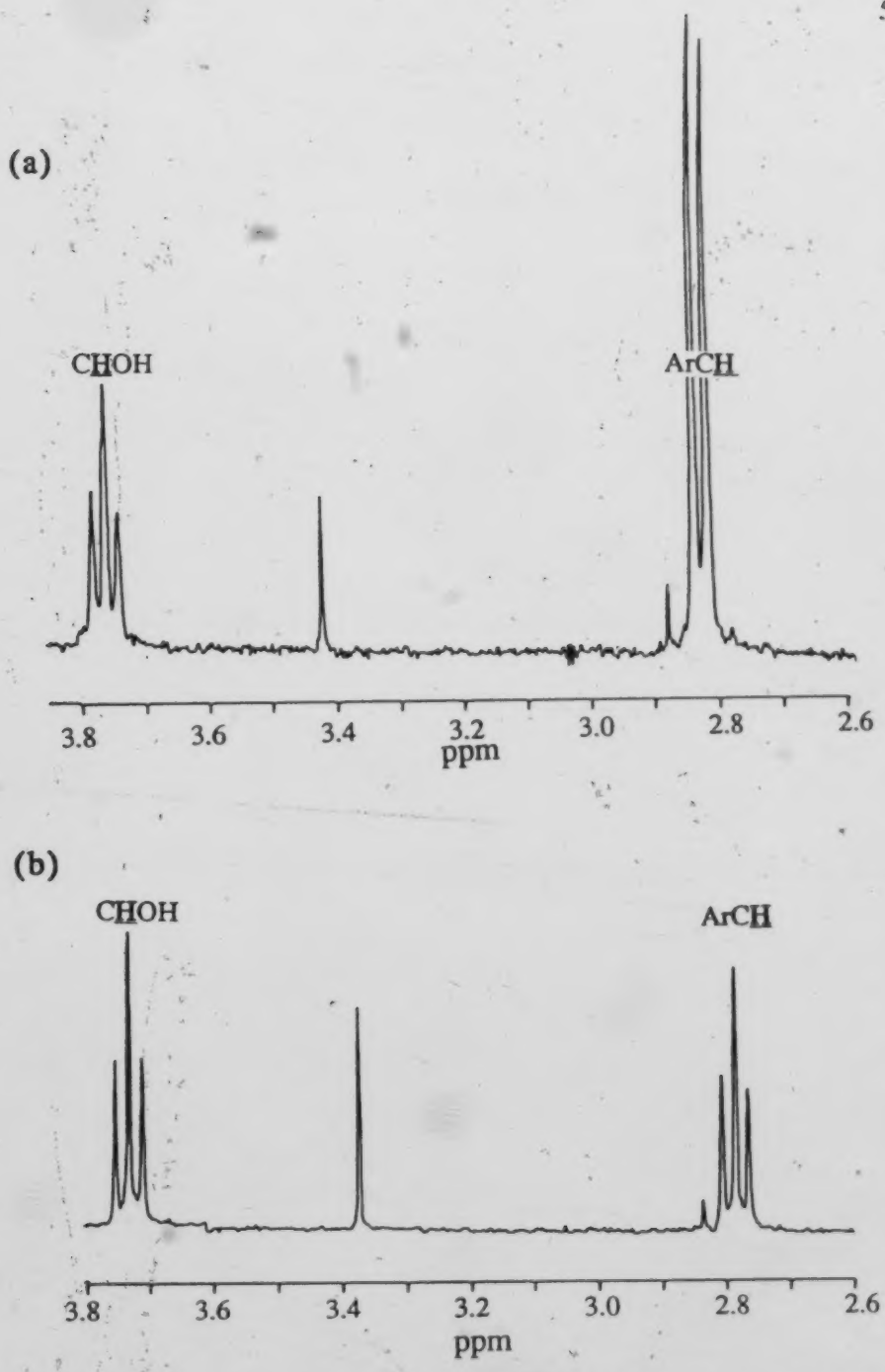


Figure 3.5. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols derived from coupled incubations of porcine kidney amine oxidase with (a) (1*R*)-[^{2}H] tyramine and (b) (1*S*)-[^{2}H] tyramine

copper amine oxidase from bovine plasma has also been shown to be substrate dependent. Specifically, benzylamine and *p*-hydroxybenzylamine oxidation occurs stereospecifically with abstraction of the pro-*S* proton, while dopamine is oxidized with apparent nonstereospecificity. Again, one of the features that distinguishes these substrates is the presence or absence of β-methylene group. It would be of interest to determine if a different isotope effects are associated with the oxidation of benzylamine compared to dopamine or tyramine by the porcine kidney amine oxidase. This may suggest that some copper amine oxidases use an alternate mechanism of oxidation for substrates with and without a β-methylene group.

Porcine kidney diamine oxidase did not catalyze the imine-enamine tautomerization, as evidenced by the presence of two triplets near 3.8 and 2.7 ppm in the spectrum of the product alcohol isolated from the solvent exchange reaction (Figure 3.6). This is a clear indication that both protons had been retained. This may be due to the lack of a correctly positioned base in the active site, to catalyze the proton abstraction a C-2, or to an ionized base, unable to accept a proton. The lack of the solvent exchange has also been noted with 2, 3-[^{3}H]putrescine but a stoichiometric loss of tritium from C-2 of randomly labelled histamine was observed (14). It was suggested that the loss of tritium during the reaction may have been due to the nonenzymatic release from the aldehyde product, imidazole acetaldehyde (24). It would be of interest to examine the stereochemistry of the tritium loss from C-2 of histamine to determine if the reaction is indeed enzyme mediated or due to the chemical reactivity of the aldehyde. The imidazole ring of histamine may alter the substrate conformation in the active site in a manner not duplicated by an aromatic ring or by an aliphatic amine. It is notable that phenethylamine derivatives are relatively poor substrates for porcine kidney diamine oxidase with turnover rates of approximately 1/10 that of aliphatic diamines (25), and that histamine has very high affinity for the enzyme, as concentrations in the millimolar range cause significant inhibition (14). This suggests that the imidazole group is required for optimal substrate binding and this may determine whether solvent exchange at C-2 occurs.

A similar stereochemical pattern of pro-*S* proton abstraction at C-1 and the absence of the solvent exchange reaction was noted for the pea, chickpea and soybean seedling amine oxidases (23). Within the copper amine oxidases, there appears to be good correlation between pro-*S* proton abstraction at C-1 and the lack of the solvent exchange pathway with aromatic or aliphatic substrates. In addition, there are a number of other similarities between these enzymes and the porcine kidney diamine oxidase. There is strong evidence that 2,4,5-trihydroxyphenylalanine, or TOPA, is the cofactor in all of these enzymes except chickpea (26). While these amine oxidases display latitude in the substrate

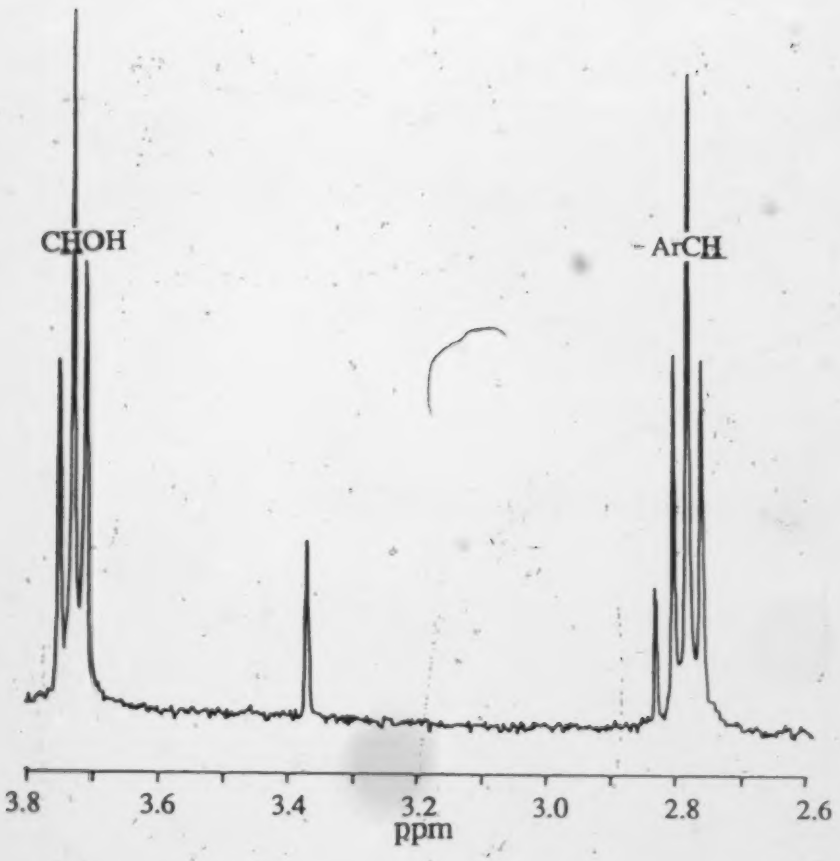


Figure 3.6. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohol derived from coupled incubation with [1,1-^{1}H] tyramine in $^{2}H_2O$ by porcine kidney amine oxidase

structure required for catalysis, they may truely be classified as diamine oxidases, since the aliphatic diamines, putrescine and cadaverine, have been shown to be the best substrates (27, 28). These factors indicate a degree of evolutionary relatedness between the pea and soybean seedling enzymes and the porcine kidney enzyme. It is somewhat curious that this mammalian enzyme should appear to be more closely related, mechanistically, to plant enzymes, rather than enzymes from other animal tissues.

Sheep Plasma Amine Oxidase

Sheep plasma amine oxidase was obtained with a higher specific activity than used in an earlier stereochemical study (29). Previously, the low specific activity enzyme exhibited an apparent nonstereospecificity in proton abstraction at C-1 of tyramine. Somewhat more complex ^{1}H-NMR spectra were obtained from the alcohol products of (1*R*)-[^{2}H] and (1*S*)-[^{2}H] tyramine compared to the stereospecific enzymes, as two alcohol products which differed by the retention or absence of deuterium at C-1, were isolated. A similar pattern was noted in this work with the higher specific activity enzyme (Figure 3.7). When (1*R*)-[^{2}H] tyramine was used as the starting material, the major product of the reaction was monoprotonated alcohol. This is evident from the NMR spectrum, exhibiting a major triplet and doublet, near 3.8 and 2.7 ppm respectively. These signals overlap minor triplets at the same chemical shifts, originating from fully protonated product. The signals from the major product containing deuterium is shifted approximately 0.02 ppm. Similar results were obtained with (1*S*)-[^{2}H] tyramine as a starting material. This is unlike a truely nonstereospecific reaction, however, in that significantly less than 50% of the deuterium was lost from each substrate as would be expected for random cleavage. A similar pattern of apparent nonstereospecificity has been noted with bovine plasma amine oxidase, and attributed to multiple binding modes coupled with large and differential deuterium isotope effects for C-1 hydrogen bond cleavage in each mode. This also appears to be a plausible explanation for the results seen with the sheep plasma amine oxidase.

Alternatively, the results could also be attributed to the presence of two enzymes, with opposite stereochemistries. In order to accomodate this second scenario, one enzyme would require a substantial isotope effect for C-1 proton cleavage which would limit its activity with one of the chiral substrates. However, as noted above, similar results were obtained with a low (0.013 U/mg protein) and high (0.13 U/mg protein) specific activity preparation, as well as with the two major isoforms of the enzyme (Table 3.2). It is unlikely that two amine oxidase activities would partition in the same ratios during the chromatographic steps used to obtain these activities. In addition, a single band of activity was obtained when the enzyme isoforms were assayed on native polyacrylamide gels (see

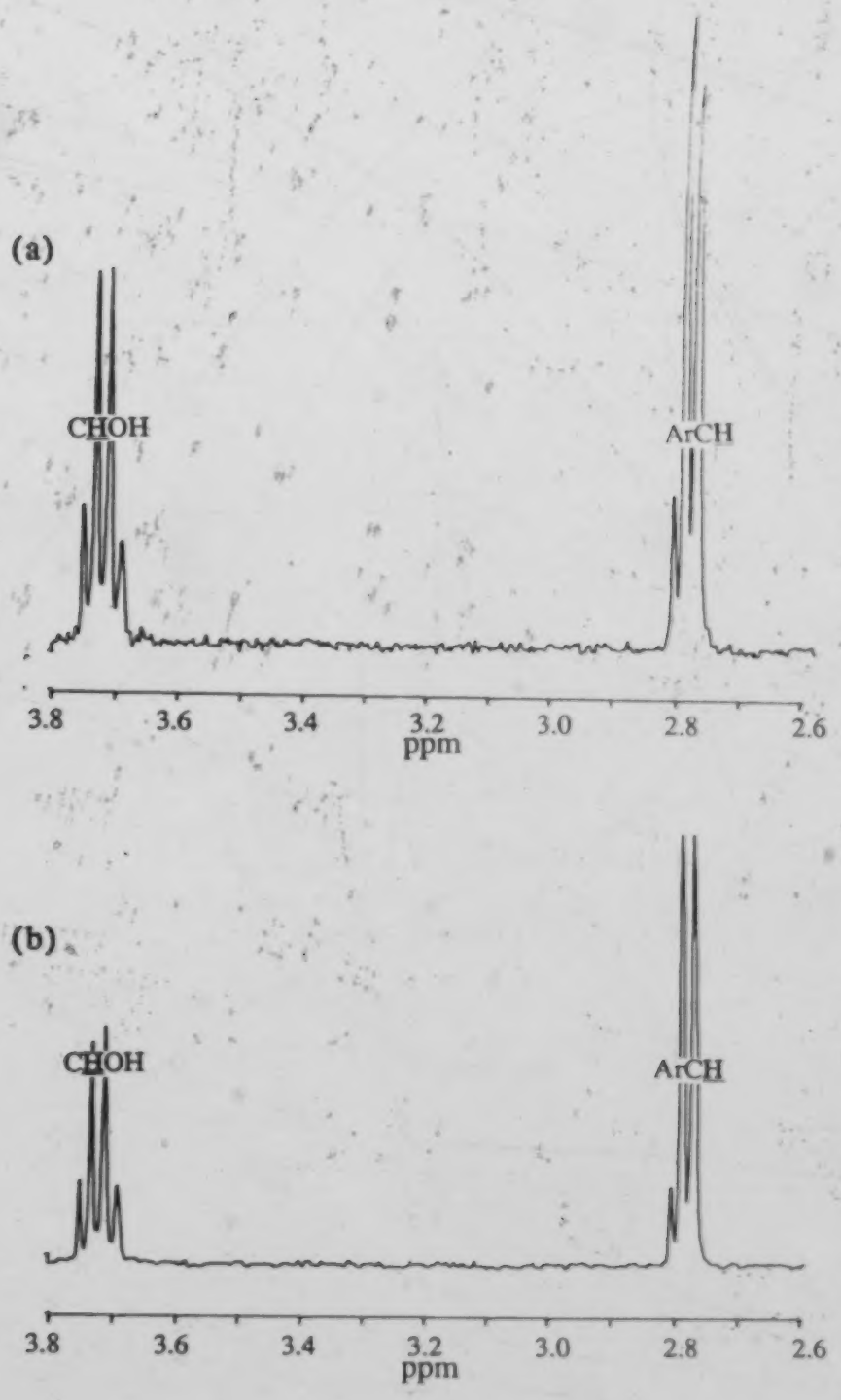


Figure 3.7. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols derived from coupled incubations of sheep plasma amine oxidase with (a) (1*R*)-[^{2}H] tyramine and (b) (1*S*)-[^{2}H] tyramine

Chapter 2). This supports the suggestion that the sheep plasma amine oxidase exhibits mirror image binding modes for tyramine, as reported for the bovine plasma amine oxidase with dopamine and tyramine (4, 5). While a similar stereochemical pattern has also been

Table 3.2 Sheep plasma amine oxidase stereochemistry with (1*R*)-[^{2}H] and (1*S*)-[^{2}H]tyramine

Enzyme Form	Monodeuterated Alcohol Products (% of total product isolated)	
	(1*R*)-[^{2}H] Tyramine	(1*S*)-[^{2}H] Tyramine
Low Specific Activity 0.013 U/mg protein[a]	72	89
High Specific Activity 0.13 U/mg protein[b]	78	93
Isoform. A 0.10 U/mg protein[b]	77	88
Isoform C 0.09 U/mg protein[b]	77	83

a Ref. 29

b This work; Isoforms identified as for Figure 2.4

observed using tyramine as a substrate for rabbit plasma amine oxidase (23), it is not typical of all plasma enzymes. Porcine plasma amine oxidase, for example, catalyzes the abstraction of the pro-*R* proton at C-1 of all substrates examined to date (5)

The preference for each stereochemical mode of proton abstraction by sheep plasma amine oxidase can be determined from the ratio of monodeuterated to fully protonate product. A ratio of 8.5 for pro-*S* and 3.4 for pro-*R* proton abstraction was calculated, suggesting that the isotope effect for the pro-S proton abstraction is larger than that for the pro-R proton abstraction. This pattern is consistent for all enzymes that exhibit dual binding modes. As noted earlier, a larger isotope effect for pro-*S* proton abstraction compared to pro-*R* has been suggested to indicate that the active site of the enzyme

accomodates the pro-*S* mode more easily (22). This allows the isotope effect for pro-*S* proton abstraction to be more fully expressed than that for pro-*R* abstraction.

Previously, sheep plasma amine oxidase was shown to catalyze the solvent exchange pathway with tyramine, with slightly less than complete deuterium incorporation (5). This result was confirmed in this work with the higher specific activity enzyme. The NMR spectrum of the *p*-hydroxyphenethyl alcohol from the solvent exchange experiment is given in Figure 3.8, and shows good incorporation of deuterium as evidenced from the doublet for the C-1 signal near 3.8 ppm and a triplet for the C-2 signal near 2.7 ppm. Slight variations in the extent of incorporation were noted, as 89 to 92% monodeuterated alcohol was obtained from the higher specific activity enzyme. The remainder of the alcohol product was the diprotonated species, with no evidence of a dideuterated product being formed. The small variation in the amount of incorporation of deuterium may be the result of slight changes in the experimental protocol, including minor fluctuations in the temperature or pH of the reaction. As well, the error associated with calculating deuterium incorporation based on integrations of NMR signals was approximately 5%. Both bovine and porcine plasma amine oxidase also catalyze this solvent exchange process, although each enzyme differs in the extent of deuterium incorporation. For the bovine enzyme, 0.8 or 1.1 deuterium atoms were incorporated while the product alcohol from the porcine enzyme contained 1.2 deuterium atoms (5). There is competition between the main hydrolytic pathway and this secondary pathway that is expressed as variation in the extent of deuterium incorporation. Small changes in the active site conformation may alter the competition between the pathways and the amount of deuterium incorporation exhibited by different enzymes. As noted earlier, this stereochemical information does not distinguish between a one base or two base mechanism for proton abstraction at C-1 and C-2. However, evidence suggests that a single base is utilized by bovine plasma amine oxidase (22, 30). The similarity of stereochemistry of bovine and sheep plasma enzymes suggests that the sheep amine oxidase may also utilize a single base.

In summary, the stereochemical pattern of tyramine oxidation and solvent exchange into C-2 can be used to distinguish the semicarbazide sensitive amine oxidase from the copper containing enzymes examined to date. The stereochemistry of C-1 abstraction is pro-*S*, similar to pea seedling, soybean seedling and porcine kidney diamine oxidase. However, semicarbazide sensitive amine oxidase catalyzes the solvent exchange reaction with tyramine, which these copper amine oxidases do not. These observations support our suggestion that the SSAO may be a distinct class of enzyme. As well, the results seen with the sheep plasma and pig kidney enzymes concur with the diverse stereochemical patterns noted for the copper amine oxidases. It is unclear why such diverse stereochemical

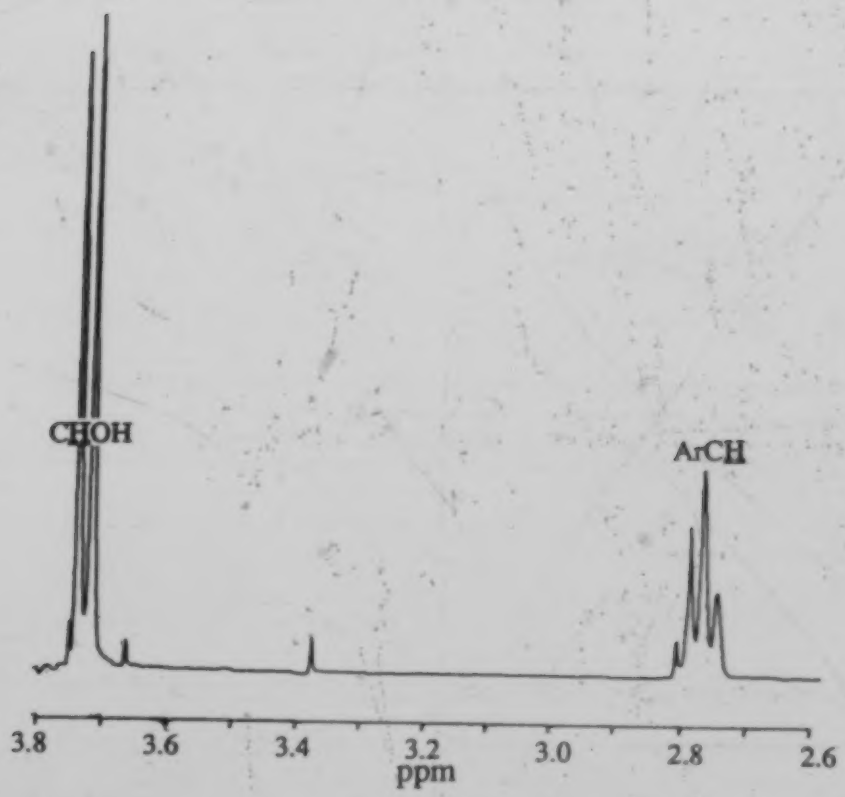


Figure 3.8. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohol derived from coupled incubation with [1,1-^{1}H] tyramine in $^{2}H_2O$ by sheep plasma amine oxidase

patterns should exist within the copper amine oxidases, since the cofactor of these enzymes and chemistry of reaction are similar. Of particular interest, is the observation that plasma enzymes from different animals do not exhibit the same stereochemistry. The analysis of a crystal structure and amino acid sequences of the SSAO and copper amine oxidases would aid in understanding their mechanism more fully.

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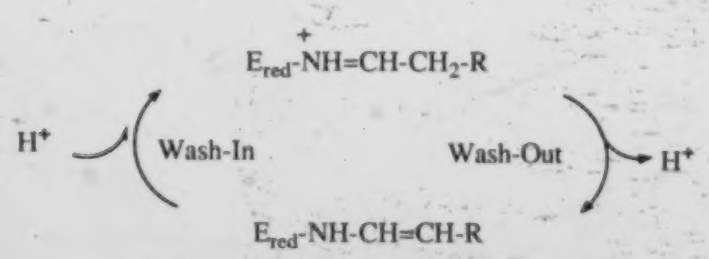
Chapter 4. Stereochemistry of Solvent Exchange Reaction of the Semicarbazide Sensitive and Sheep Plasma Amine Oxidases

Introduction

A unique pattern of oxidation of tyramine was observed for bovine and porcine semicarbazide sensitive amine oxidases (SSAO), compared to other copper amine oxidases, as described in Chapter 3. Both SSAO enzymes were found to catalyze the removal of the pro-*S* proton at C-1 of tyramine and to incorporate a proton from solvent at the C-2 position. Alternatively, the porcine kidney amine oxidase was typical of the copper amine oxidases that have been observed to catalyze the abstraction of the pro-*S* proton at C-1, lack the solvent exchange pathway (1). This suggests that a basic mechanistic difference exists between SSAO and copper dependent amine oxidases. The sheep plasma amine oxidase, however, was found to catalyze the same apparent nonstereospecific proton abstraction at C-1 as the bovine plasma amine oxidase (2) and to exhibit the exchange of a proton with solvent at C-2. It was of interest then, to characterize the stereochemistry of the imine:enamine pathway for the SSAO enzymes and the sheep plasma amine oxidase and compare the results to those reported for other copper dependent amine oxidases.

The exchange of protons at C-2 of amine substrates is a side reaction that occurs off the main catalytic pathway, presumably through an imine intermediate (Scheme 4.1).



Scheme 4.1 Solvent Exchange Pathway

It is the sum of two reactions. First, there is the loss of a proton from C-2 of the enzyme bound imine intermediate, referred to as wash-out. Following this, reprotonation from solvent of the resulting enamine, referred to as wash-in, completes the pathway. The stereochemistry of each of the reactions of solvent exchange may be observed independently. The stereochemistry of the wash-out reaction can be determined by following the loss or retention of a hydrogen isotope from C-2 chirally labelled amines (2).

Alternatively, the wash-in reaction can be observed if the amine oxidase reaction is carried out in a solvent containing a different hydrogen isotope than is present at C-2 of the amine (3). The stereochemistry of the wash-in can then be determined by configurational analysis of the new hydrogen isotope at C-2.

Bovine plasma amine oxidase was first reported to catalyze the abstraction of tritium from the C-2 position of dopamine by Lovenberg and Beaven (4). It was also observed that monoamine oxidase from guinea pig livers did not exchange protons at C-2 with solvent which suggested a mechanistic difference between the copper and flavin dependent enzymes. Since then, it has been shown that even within the copper amine oxidases, the expression of the imine:enamine pathway occurs inconsistently and is not a required step in amine oxidation. For example, the porcine kidney diamine oxidase (Chapter 3), and the pea, chick pea and soybean seedling amine oxidases do not exhibit the C-2 exchange pathway although these enzymes produce the usual products of the amine oxidase reaction (1).

Since the initial report of the C-2 solvent exchange pathway observed for the bovine plasma amine oxidase, stereochemical studies of the process were carried out which led to a more complete understanding of the mechanism of this enzyme. The exchange reaction was characterized by monitoring the incorporation and loss of tritium from C-2 of dopamine. It was expected that the two reactions would follow equivalent mechanisms in the forward and reverse directions, but experimental evidence indicated that enamine intermediates of each reaction were not equivalently bound. The wash-out of tritium from dopamine by the bovine enzyme was reported to be an apparent nonstereospecific reaction, characterized by two binding modes with opposite but absolute stereochemistries (2). However, the wash-in reaction in tritiated water was found to be stereospecific, with pro-*R* incorporation of label at C-2 (3). This information, along with details of the sterochemical mode of proton abstraction at C-1 and the associated isotope effects, was used to construct a working model of the bovine plasma amine oxidase active site. The model supported the theory that a single base catalyzed the proton abstraction at C-1 and the exchange of a proton at C-2. These studies have stimulated further investigations into the stereochemistry of the C-2 exchange pathway catalyzed by amine oxidases. The stereochemistry of the C-2 solvent exchange of the bovine and porcine plasma amine oxidase with tyramine was recently examined (5). The porcine plasma amine oxidase was found to catalyze a nonstereospecific wash-out and wash-in reaction with tyramine, while the stereochemistry of the bovine enzyme was found to be nonstereospecific for wash-out and pro-*R* for wash-in with tyramine, as had been reported for dopamine.

In the present study the stereochemistry of wash-out and wash-in reactions were determined for bovine and porcine aortic SSAO and sheep plasma amine oxidase. Wash-out stereochemistry was determined using C-2 chirally deuterated amines obtained from the bovine plasma amine oxidase wash-in reaction; the stereochemistry of the wash-in reaction was determined using *S*-(+)-*O*-acetylmandelic acid as a chiral derivatizing agent. The stereochemistry of the C-2 proton exchange supports other observations that the SSAO enzymes are a unique class of enzyme from other amine oxidases, including the copper amine oxidases and the flavin dependent monoamine oxidases. Sheep plasma amine oxidase displayed stereochemical behaviour at C-2 of tyramine similar to that of the bovine plasma amine oxidase, as expected. Therefore, it is likely that a single base acts on C-1 and C-2 of tyramine, as suggested for the bovine plasma amine oxidase.

Materials and Methods

All chemicals were of reagent grade unless otherwise specified and all protonated solvents were distilled before use. Deuterated solvents were obtained from Merck, Sharp and Dohme (99.95% ^2H), or General Intermediates of Canada (99.9% ^2H). [2,2-$^2H_1$]-Tyramine HCl (98 atom% $^2H_1$) was obtained from Merck, Sharp and Dohme. Reverse osmosis water that was passed through an anion exchange, cation exchange, carbon and 0.45 µm cartridges was used for all chemical solutions and buffers. Thin layer chromatography plates were Kieselgel 60 F_{254}, 0.2 mm, from E. Merck, Darmstadt.

Enzymes

Horse liver alcohol dehydrogenase, catalase and bovine plasma amine oxidase were obtained from Sigma. SSAO from porcine and bovine aortic tissue and sheep plasma amine oxidase were isolated as outlined in Chapter 2. Pea seedling amine oxidase was a gift from A. Coleman.

^1H-NMR Spectroscopy

^1H-NMR spectra were measured at 360 MHz on a Bruker WM-360 instrument at ambient temperature (22 + 1°C). The spectra of *p*-hydroxyphenethyl alcohols were obtained in $(^2H_3C)_2CO$:^2H_2O (4:1), with the residual H^2H_2C signal set at 2.19 as an internal reference standard.[1] Optimization of the conditions for obtaining NMR spectra was determined by Dr. O. Hindsgaul[1] . Spectra of tyramine hydrochlorides were obtained in ^2H_2O using the residual H^2HO signal at 4.81 as an internal reference standard. Spectra of

[1] Dept. of Chemistry, University of Alberta

S-(+)-*O*-acetylmandelate esters were obtained in $C_6{}^2H_6$ using residual $C_6{}^2H_5{}^1H$ signal at 7.15 as an internal standard. Spectra were accumulated into 16 K of computer memory using a 45° pulse with a 7 second relaxation delay between pulses for ^2H_2O-containing solvents and a 2 second relaxation delay for organic solvents.

Coupled Amine Oxidase Reaction

(2*R*)-[^{2}H] and (2*S*)-[^{2}H] *p*-Hydroxyphenethyl Alcohols

Coupled amine oxidase reactions were carried out as described by Battersby and coworkers (6) with some modifications. Two units of bovine plasma amine oxidase were incubated in 6 mL of 30 mM phosphate buffer, pH 7.6 containing 60 µmol of [2,2-^{2}H]tyramine, or in deuterated buffer, pD 7.1 with 60 µmol [2,2-^{1}H]tyramine for the production of (2*S*)-[^{2}H] and (2*R*)-[^{2}H] *p*-hydroxyphenethyl alcohols, respectively. Coupling reagents, including 150 µmol of NADH, 11 units of alcohol dehydrogenase and 80,000 units of catalase, were added. Incubations were carried out at 37°C and allowed to proceed for 3 to 6 hours, or overnight. After completion of the reaction, enzyme incubations were extracted 3 times with 25 mL of ethyl acetate. Successive extracts were concentrated to dryness under reduced pressure and flash chromatographed on 2 gm silica (60-100 mesh, BDH) with ethyl acetate: hexane (2:1) as a solvent. After drying, samples were analyzed by ^{1}H-NMR to determine the extent of solvent incorporation at C-2.

Wash-Out of Deuterium from (2*R*)-[^{2}H] and (2*S*)-[^{2}H] Tyramines by Amine Oxidases

Amine oxidase (0.1 unit), 2.5 µmol (2*R*)-[^{2}H] or (2*S*)-[^{2}H] tyramine, 2.5 units alcohol dehydrogenase, 15,000 units catalase, 15 µmol NADH were reacted in 0.5 mL 100mM phosphate buffer, pH 7.6. Incubations, product isolation and analysis were carried out as described above for (2*R*)-[^{2}H] and (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohols.

Wash-In of Solvent into C-2 of *p*-Hydroxyphenethyl Alcohols

Amine oxidase (0.3 units) was incubated in 6 mL of 30 mM phosphate buffer, pH 7.6 containing 15 µmol of [2,2-^{2}H]tyramine, or in deuterated buffer, pD 7.2 with 15 µmol [2,2-^{1}H]tyramine, 40 µmol of NADH, 6 units of alcohol dehydrogenase and 30,000 units of catalase. Incubations, product isolation and analysis were carried out as described above for (2*R*)-[^{2}H] and (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohols.

Chemical Conversion of [2-^{2}H]-p-Hydroxyphenylethyl Alcohol to [2-^{2}H]-Tyramine

The following procedure for the chemical conversion of [2-^{2}H]-*p*-hydroxyphenylethyl alcohol to [2-^{2}H]-tyramine was that of Farnum and Klinman (3), modified by Dr. O. Hindsgaul. The quantities of reagents given below are for the conversion of 5 mg of alcohol to tyramine.

Protection of [2-^{2}H]-*p*-Hydroxyphenylethyl Alcohol

A 2 molar excess of 1,8-diazabicyclo-[5.4.0]undecene-7 (DBU) (11.6 µl) and benzyl chloride (9 µl) were added to a screw cap test tube with the deuterated alcohol (5 mg) dissolved in 1 mL of methanol. The mixture was capped and stirred for 18 hours at 65°C. A second 2 molar excess of DBU and benzyl chloride were added if neccessary. The reaction was followed by silica TLC using ethyl acetate:n-hexane:95% ethanol (10:10:1) as a solvent (Rf=0.67). After cooling, 1 mL of 1N NH_4OH was added and the reaction was stirred for 30 minutes. The benzylated alcohol was extracted into CH_2Cl_2, washed with water, 1N HCl, water, and dried under reduced pressure. ^{1}H NMR in $CDCl_3$ (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 2.7 (t, 1H), 3.8 (d, 2H), 5.0 (s, 2H), 6.9 (d, 2H), 7.1 (d, 2H), 7.4 (m, 5H).

Conversion of 4-Benzyloxyphenylethyl Alcohol to Tolylsulfonyl Ester

The benzylated adduct was dissolved in 0.5 mL dry CH_2Cl_2 and 40 µmol 4-dimethylaminopyridine (DMAP) (4.86 mg) in 250 µl CH_2Cl_2 and 30 µmol tolylsulfonyl chloride (5.73 mg) in 250 µl CH_2Cl_2 were added. The reaction mixture was stirred at ambient temperature for several hours and a second addition of reagents was added if required. The reaction was followed by silica TLC using ethyl acetate:n-hexane:95% ethanol (10:10:1) as a solvent (Rf=0.85). A 0.5 mL volume of pyridine:water (4:1) was added and stirred for 30 minutes. The tosylated product was extracted into CH_2Cl_2, washed with water, 1 N HCl and water adjusted to pH 9.0 with bicarbonate. The adduct was filtered through paper and dried under reduced pressure. ^{1}H-NMR in $CDCl_3$ (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 2.5 (s, 3H), 3.0 (t, 1H), 4.2 (d, 2H), 5.4 (s, 2H), 6.9 (d, 2H), 7.1 (d, 2H), 7.3 (d, 2H), 7.4 (m, 5H), 7.7 (d, 2H).

Formation of Phthalimide Derivative

A 5 fold excess (w/w) of potassium phthalate (25 mg) to tosylated adduct was added in 0.6 mL dry N,N-dimethylformamide (DMF) to the tosylated adduct. The mixture was stirred at 60^{0}C over night, the phthalimide adduct was extracted into CH_2Cl_2 and

washed with ice water, 1N NaOH with ice, and then water until neutral. The product was filtered through paper and dried under reduced pressure. The product was isolated by chromatography on a 5 gram silica flash column using CH_2Cl_2:95% ethanol (98:2) as solvent. ^{1}H-NMR in $CDCl_3$/CD_3OD (50/1) (internal reference $CHCl_3$, δ 7.24) showed the following (δ in ppm): δ 3.0 (t, 1H), 4.0 (d, 2H), 5.1 (s, 2H), 6.9 (d, 2H), 7.2 (d, 2H), 7.4 (m, 5H), 7.7 (m, 2H), 7.9 (m, 2H).

Hydrazinolysis of Phthalimide Derivative to Protected Amine

The phthalimide adduct was dissolved in 1 mL of 95% ethanol, with 100 µL of hydrazine hydrate, and the mixture was stirred over night at 65^{0}C. The product was dried under reduced pressure, dissolved in CH_2Cl_2 and washed 2 times with 1 N NaOH and 2 times with water, and dried under reduced pressure. The reaction was followed by silica TLC using isopropanol:H_2O:NH_4OH (7:1:1) as a solvent (Rf=0.64). ^{1}H-NMR in CD_3OD (internal reference CD_2HOD, δ 4.78) showed the following (δ in ppm): δ 2.7 (t, 1H), 2.9 (d, 2H), 5.1 (s, 2H), 6.9 (d, 2H), 7.1 (d, 2H), 7.3(m, 5H).

Deprotection of 4-Benzyloxyphenylethyl amine to tyramine

The material was dissolved in 3 mL of 95% ethanol with 5 drops of 1 N HCl and 3-5 mg of 5% palladium on charcoal (BDH). Hydrogenolysis was carried out at ambient temperature and H_2 flow of 5 psi overnight. The reaction was followed by silica TLC using isopropanol:H_2O:NH_4OH (7:1:1) as a solvent (Rf=0.60). At completion of the reaction, the catalyst was removed by filtration with methanol and dried. No further purification was required. Overall yield of the reaction was 25 to 45%. ^{1}H-NMR showed retention of deuterium at C2 with no loss or scrambling during the chemical conversion. ^{1}H-NMR in D_2O (internal reference HDO, δ 4.81) showed the following (δ in ppm): δ 3.1 (t, 1H), 3.3 (d, 2H), 6.9 (d, 2H), 7.2 (d, 2H).

Formation of S-(+) -O-Acetylmandelate Esters

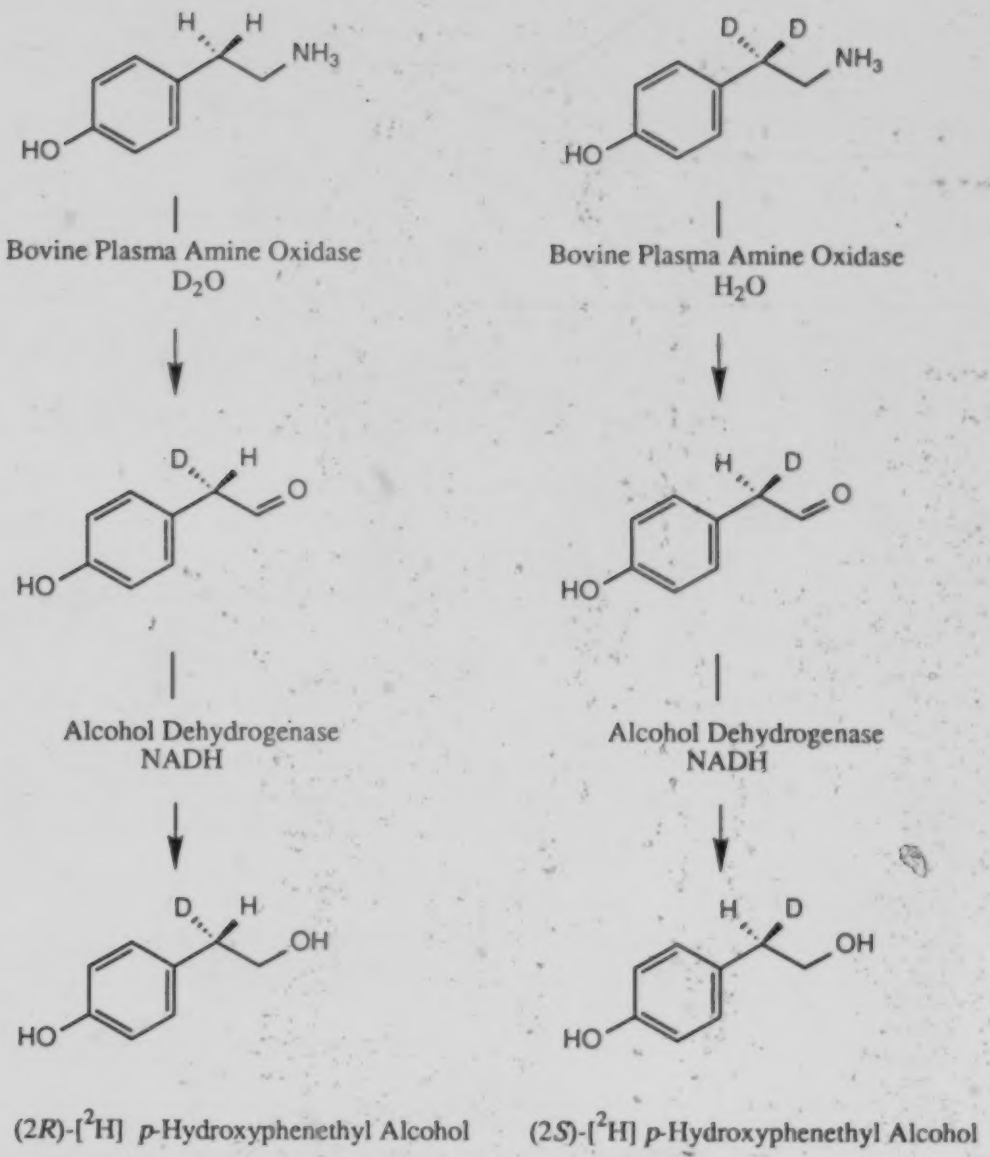
The derivatization and purification protocol were modified from Parker (7) and Whitesell and Reynolds (8) for reaction on a scale of 1 mg or less. To a solution of 7 µmol tyrasol, 15 µmol *S*-(+)-*O*-acetylmandelic acid and 1 µmol 4-dimethylaminopyridine in 1 mL dichloromethane at -10°C was added 15 µmol dicyclohexylcarbodiimide in 0.5 mL of dichloromethane. A white precipitate of dicyclohexylurea was observed to be formed within one minute of addition of dicyclohexylcarbodiimide. The reaction was allowed to proceed for two hours, stirring, while the temperature of the reaction gradually increase to

room temperature. The reaction was followed by silica TLC with ethyl acetate:n-hexane (1:2) as solvent (Rf=0.40). The solution was washed successively with 25 mL portions of 0.5 N HCl, 2 N Na_2CO_3 and a saturated salt solution. The sample was dried under reduced pressure, resulting in a white solid. The dry material was solubilized in ethyl acetate:n-hexane (1:2) and the product isolated by chromatography on a 2 gm flash silica column with the same solvent. The ester product, which was a liquid, was analyzed by ^{1}H-NMR.

Results and Discussion

(2R)-[^{2}H] and (2S)-[^{2}H] Tyramine

Determining the stereochemistry of the wash-out reaction of the semicarbazide sensitive amine oxidases and sheep plasma amine oxidase required the use of amines with known configuration of deuteration at C-2. These chiral amines were obtained by exploiting the known specificity of bovine plasma amine oxidase wash-in reaction, and the commercial availability of the enzyme. The reaction is reported to proceed with pro-*R* incorporation of solvent into the C-2 position of dopamine (3). From tritiated solvent, 95% of C-2 pro-*R* labelled alcohol was obtained. Similarly, bovine plasma amine oxidase was found to catalyze pro-*R* incorporation of 1.1 or 0.8 deuterium atoms at C-2 of tyramine (5). Therefore, the reaction of bovine plasma amine oxidase in a deuterated buffer and [2,2-^{1}H] tyramine, coupled with alcohol dehydrogenase and NADH, resulted in (2*R*)-[^{2}H] *p*-hydroxyphenethyl alcohol while the reaction in protonated buffer with [2,2-^{2}H] tyramine results in (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohol (Scheme 4.2). The ^{1}H-NMR spectra of the alcohols from these incubations are shown in Figure 4.1. Integration of the spectra of (2*R*)-[^{2}H] *p*-hydroxyphenethyl alcohol indicated that 96% of the product was the monodeuterated species while 4% was diprotonated. The doublet near 3.8 ppm and the large triplet near 2.7 ppm are attributed to the C-1 and C-2 signals originating from the monodeuterated product. These signals are shifted slightly upfield from smaller triplets at each of these ppm, that are attributed to the diprotonated species. Integration of the spectra of (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohol from the wash-in reaction in protonated buffer indicated that 72% of the product from the reaction was monodeuterated, 8% was



Scheme 4.2. Enzymatic formation of (2*R*)-[^{2}H] and (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohols using bovine plasma amine oxidase

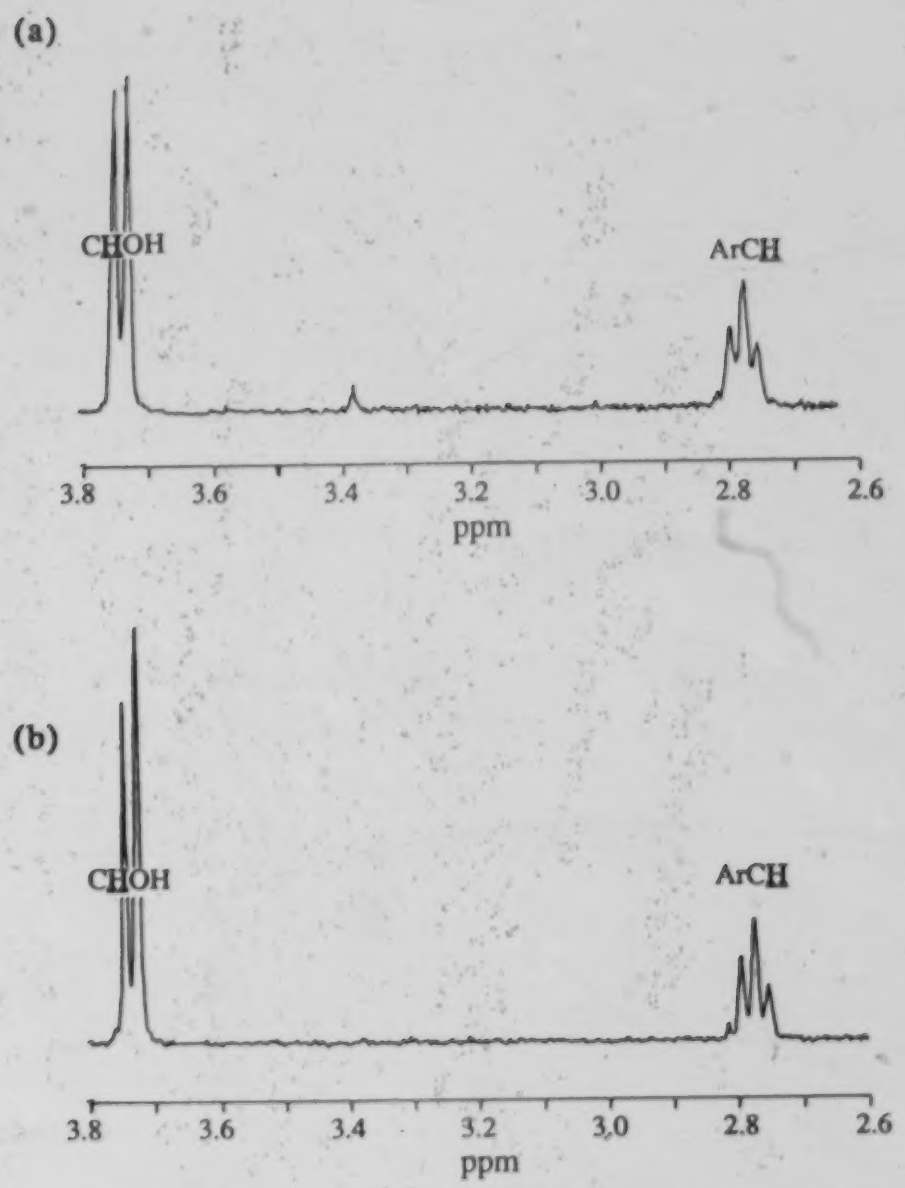
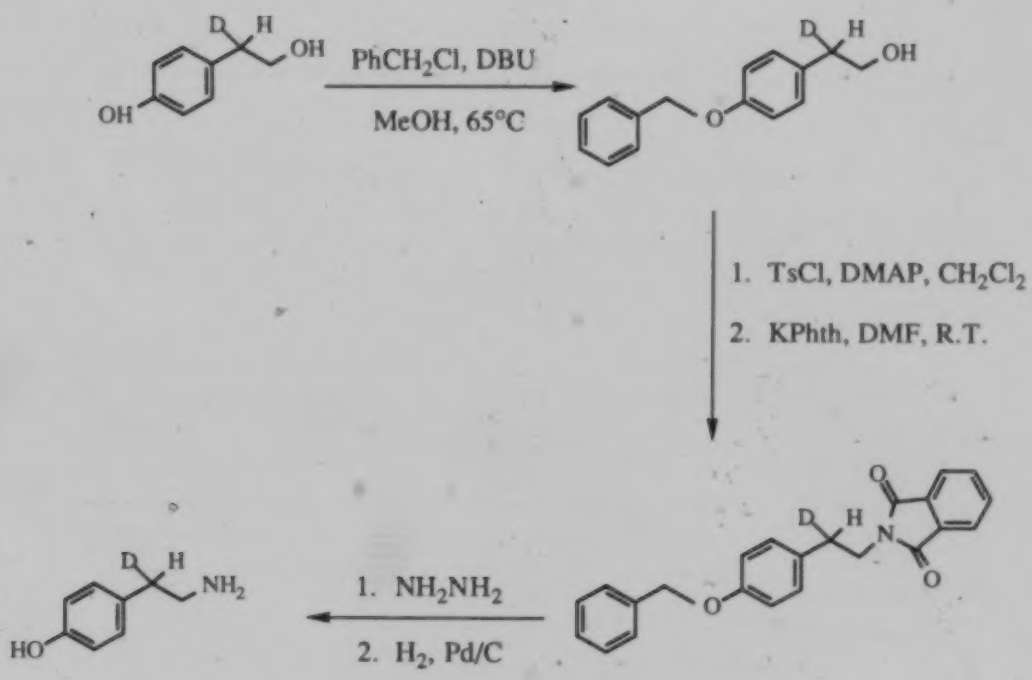


Figure 4.1. ^{1}H-NMR spectra of C-2 deuterated *p*-hydroxyphenethyl alcohols derived from coupled incubations of bovine plasma amine oxidase (a) (2*R*)-[^{2}H] and (b) (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohols

diprotonated and 20% was dideuterated. The enzyme appears to be less stereospecific when the wash-out reaction involved the removal of deuterium from C-2, rather than incorporation of deuterium from solvent. It was noted that, in some cases, an altered ratio of dideuterated, monodeuterated and diprotonated alcohols were produced in incubations left overnight, compared to incubations carried out for three to six hours. This may have been due to a reduced efficiency of the coupling reagents, and could be avoided by reducing the incubation time.

Previously, confirmation that the wash-in reaction catalyzed by bovine plasma amine oxidase was indeed pro-*R* for both reactions was established by chemically converting the alcohols to amines, and then reacting the amines with a mixed function oxidase, dopamine-β-hydroxylase (E.C.1.14.17.1) (3, 5). This enzyme catalyzes the abstraction of the pro-*R* proton from C-2 of dopamine (9), amphetamine (10) and tyramine (5) followed by the insertion of a hydroxyl group. Therefore, it was expected that deuterium would be lost from the (2*R*)-[^{2}H] tyramine and retained in the (2*S*)-[^{2}H] tyramine, as was found. In this work, the stereochemical uniformity of the bovine plasma amine oxidase wash-in reaction in deuterated and protonated solvent was confirmed by derivatizing the C-2 deuterated alcohols with *S*-(+)-*O*-acetylmandelic acid. Further detail of this analysis will be described later under the discussion of the wash-in reaction of the SSAO and sheep plasma amine oxidases.

The C-2 chirally deuterated alcohols obtained from the bovine plasma amine oxidase reaction in deuterated or protonated solvent were chemically converted to amines using the protocol outlined in Materials and Methods (Scheme 4.3). This procedure was modified from that of Klinman and coworkers (3) by using the base, DBU, during the benzylation of the ring hydroxyl. As well, a tosylated derivative was formed as an intermediate before the formation of the phthalimide derivative. Yields from the reaction, of 25 to 45%, were increased compared to those reported for the unmodified protocol of 10%. No scrambling or loss of deuterium label occurred during the conversion, as determined from integration of the ^{1}H-NMR signals of C-1 and C-2 protons of starting alcohol compared to amine product. ^{1}H-NMR spectra of the amines, given in Figure 4.2, contain the same ratio of monodeuterated to diprotonated product as observed for the original alcohols (Figure 4.1). The signals for the C-1 and C-2 protons of the monodeuterated amine are attributed to the doublet near 3.2 ppm and the triplet near 2.9 ppm, respectively. The minor signals, shifted slightly downfield are attributed to the diprotonated amine product.



Scheme 4.3. Chemical conversion of *p*-hydroxyphenethyl alcohol to tyramine

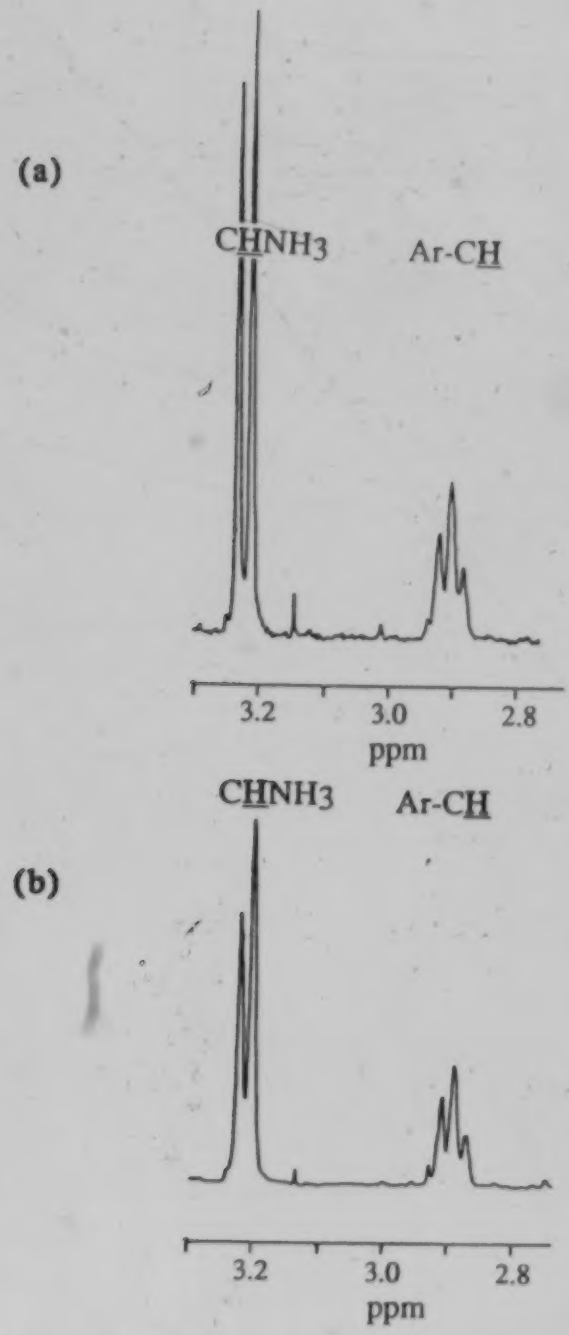


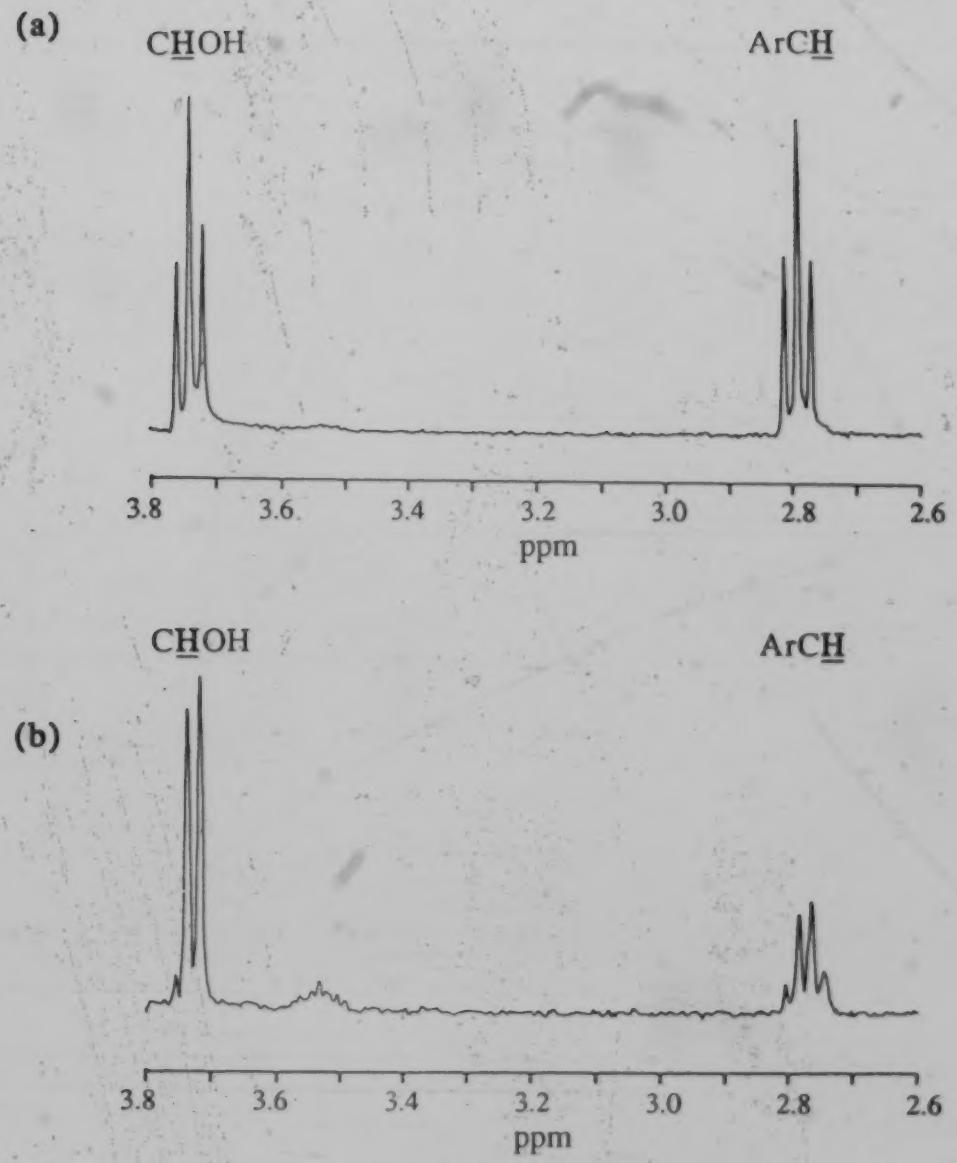
Figure 4.2. ^{1}H-NMR spectra of C-2 deuterated tyramines from chemical conversion of C-2 deuterated *p*-hydroxyphenethyl alcohols (a) (2*R*)-[^{2}H]tyramine and (b) (2*S*)-[^{2}H]tyramine

Wash-Out from the Enamine Intermediate

With the availability of chiral amines at C-2 of known configuration, the stereochemistry of the wash-out reaction of the SSAO and the sheep plasma amine oxidase reactions could be determined. The stereochemical studies were carried out in the usual manner so that amines were converted to aldehyde products by the amine oxidases. The aldehydes were then coupled directly to alcohol production using alcohol dehydrogenase and NADH. The deuterium content of the *p*-hydroxyphenyl alcohol products compared to the substrates served as a marker for the steric course of the reaction. Retention of deuterium during this reaction resulted in an ^{1}H-NMR spectrum of the alcohol consisting of a doublet for the C-1 proton signal and a triplet for the C-2 proton signal, with chemical shifts near 3.8 ppm and 2.7 ppm, respectively. A loss of deuterium from the alcohol resulted in a ^{1}H-NMR spectrum of two triplets for C-1 and C-2 proton signals with approximately the same chemical shifts.

The porcine aortic SSAO was found to catalyze the abstraction of the pro-*R* proton from C-2 of tyramine. This is evident from the ^{1}H-NMR spectra of the alcohols from the oxidation of the (2*R*)-[^{2}H] and (2*S*)-[^{2}H] tyramine, shown in Figure 4.3. The spectrum of the alcohol product of (2*S*)-[^{2}H] tyramine exhibited mainly a doublet near 3.8 ppm for the C-1 signal and the triplet near 2.7 ppm for the C-2 signal, indicating retention of deuterium. Minor signals corresponding to two triplets from fully protonated product are also present at approximately the same ppm, indicating that a small amount of deuterium had been lost in the reaction. The major signals are shifted 0.02 ppm upfield due to an α- and β-deuterium isotope effect, respectively (11). Integration of the signals indicates that 86% of the product was the monodeuterated species while 14% was the diprotonated species while the amine substrate contained 8% diprotonated alcohol product. This indicates that a small amount of the reaction followed pro-*S* specificity at C-2.

The corresponding spectrum of the alcohol product of (2*R*)-[^{2}H] tyramine is comprised of a triplet near 3.8 ppm and a triplet near 2.7 ppm , indicating that the deuterium had been lost during the reaction. A slightly reduced integration for the C-2 signal at 2.7 ppm of 1.95 protons indicated that a small amount of monodeuterated product may be present, however, the expected signal from the triplet shifted slightly upfield from the major product near 2.7 ppm was not visible. As well, the doublet for the monodeuterated signal at 3.8 ppm would be completely hidden under the triplet, but integration of the individual peaks of the triplet indicated a deviation from the expected



Figûre 4.3. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols from coupled incubations of porcine SSAO with (a) (2*R*)-[^{2}H] tyramine and (b) (2*S*)-[^{2}H] tyramine

1:3:1 ratio for the peaks. This suggested that approximately 10% of monodeuterated product was present. With these factors considered, it was estimated that 5 to 10% of the reaction proceeded with pro-*S* proton abstraction and that the majority of the reaction had followed pro-*R* specificity.

The bovine aortic SSAO also exhibited mainly pro-*R* proton abstraction from C-2 of tyramine. The ^{1}H-NMR spectra of the alcohols from the oxidation of the (2*R*)-[^{2}H] and (2*S*)-[^{2}H] tyramine shown in Figure 4.4. The spectrum of the alcohol product from (2*S*)-[^{2}H] tyramine was very similar to that from the porcine enzyme, exhibiting mainly a doublet near 3.8 ppm and the triplet near 2.7 ppm. Minor signals corresponding to two triplets from fully protonated product were also present at approximately the same ppm, indicating that a small amount of deuterium had been lost in the reaction. The major signals near 3.7 and 2.8 ppm are shifted 0.02 ppm upfield. Integration of the signals indicates that 86% of the product was the monodeuterated species while 14% was the diprotonated species, again suggesting that a small amount of the reaction had followed pro-*S* specificity as observed for the porcine SSAO.

The reaction with (2*R*)-[^{2}H] tyramine appeared to proceed with predominantly pro-*R* specificity, however this reaction was less stereospecific than the corresponding reaction with (2*S*)-[^{2}H] tyramine. The spectrum of the alcohol product of (2*R*)-[^{2}H] tyramine was composed mainly of a triplet near 3.8 ppm and a triplet near 2.7 ppm, indicating that a significant portion of the deuterium had been lost as expected. However, a signal from the triplet of the monodeuterated product was visible, shifted slightly upfield from the major signal near 2.8 ppm. As well, a reduced integration for the C-2 signal near 3.8 ppm of 1.62 protons indicated that 30% of the product was monodeuterated while 70% of the product was diprotonated. This may indicate that an isotope effect exists against pro-*R* proton abstraction at C-2, as it was expected that approximately 10% of the deuterium would be retained, similar to that retained by the porcine SSAO during the oxidation of (2*R*)-[^{2}H] tyramine. This is the first discrepancy in the stereochemistry of the bovine and porcine SSAO enzymes.

With or without an isotope effect on proton abstraction from C-2, these results indicate that both SSAO enzymes catalyze the abstraction of the pro-*R* proton at C-2 while the stereochemical mode of proton abstraction at C-1 was mainly pro-*S*. This is the same stereochemistry exhibited by the bovine plasma amine oxidase for one of the dopamine binding modes. This stereochemistry may be realized with either a syn or anti conformation, by utilizing a single base or two bases, respectively, for C-1 and C-2 proton abstraction (Scheme 4.4). A syn relationship is suggested if assumptions about the active

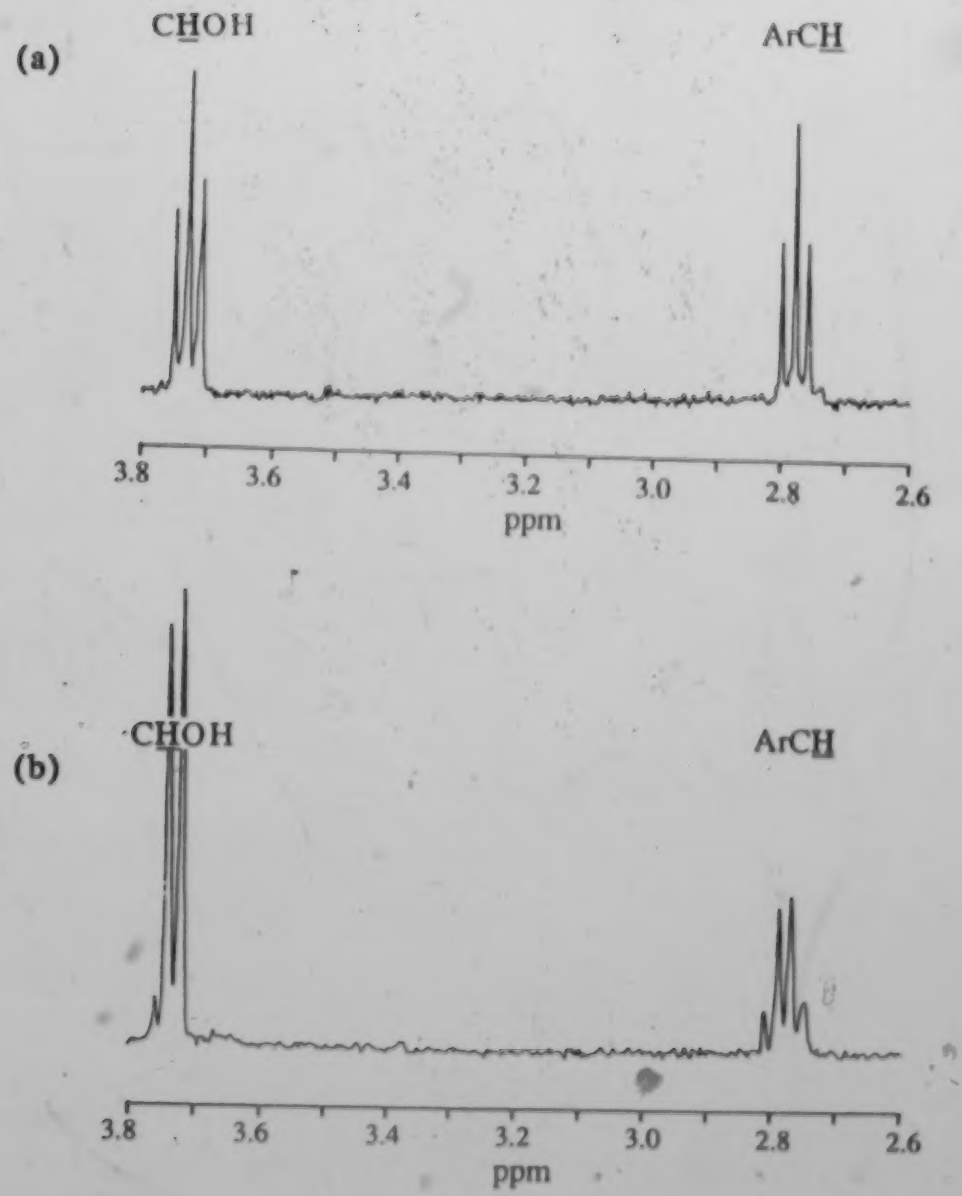
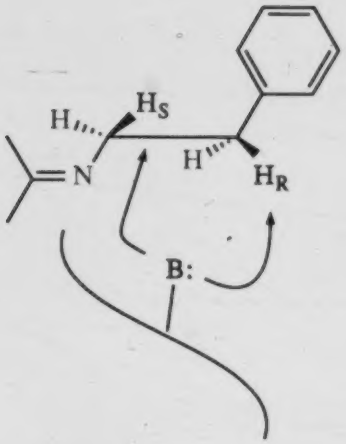
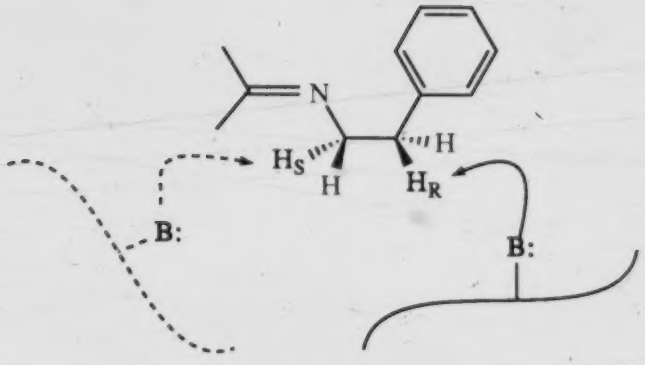


Figure 4.4. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols from coupled incubations of bovine SSAO with (a) (2*R*)-[^{2}H] tyramine and (b) (2*S*)-[^{2}H] tyramine

(a)



(b)



Scheme 4.4 Schematic representation of the active site of the semicarbazide sensitive amine oxidases conducive to (a) syn or (b) anti proton abstraction from C-1 and C-2

site conformation of the bovine plasma enzyme, described by Summers and coworkers (2) are valid for the SSAO. These researchers constructed a model which established a fixed geometry for the hydrogen and nitrogen bonds at C-1 that undergo cleavage, and accounted for the pro-*S* proton abstraction from C-1 of benzylamine and the dual binding modes of dopamine (and tyramine). Their model also limited interactions between the aromatic ring of the substrate and the pyridine ring of the cofactor, then suggested to be PQQ. However, SSAO do not have dual binding modes for tyramine (this work) or dopamine (13) since the position of the isotope did not effect the stereochemistry of the reaction. As well, the cofactor, while probably quinone in nature, does not appear to be TOPA found in the bovine plasma amine oxidase. Therefore, an anti conformation is also possible to account for the proton abstractions at C-1 and C-2. However, further information such as a pH dependency study for C-1 and C-2 proton abstraction would be required to establish this.

The pattern of C-1 and C-2 proton abstraction by the SSAO is unique when compared to the stereochemical modes of reaction of amine oxidases examined to date. All flavin-dependent monoamine oxidases have been found to exclusively catalyze the abstraction of the pro-*R* proton at C-1 (13, 14) without the solvent exchange pathway at C-2 (4). The copper amine oxidases which also catalyze the pro-*S* proton abstraction at C-1, lack the solvent exchange pathway at C-2 (1). Examples of these copper amine oxidases include those from plant (pea, soybean and chick pea seedling) and animal (porcine kidney) sources. Alternatively, the copper amine oxidase from bovine and porcine plasma which do exhibit the solvent wash-in reaction have been reported to exhibit nonstereospecificity in the wash-out reaction or in both the wash-out and wash-in reactions (5). This supports the proposal that the SSAO enzymes constitute a class of amine oxidase, distinct from the copper and flavin amine oxidases.

The sheep plasma amine oxidase catalyzed a nonstereospecific proton abstraction from C-2 of tyramine. The spectra of the alcohols from (2*R*)-[^{2}H] and (2*S*)-[^{2}H] tyramine are shown in Figure 4.5. In both cases, a mixture of monodeuterated and diprotonated product was produced. The signal near 3.8 ppm attributed to the C-1 protons is composed of a triplet superimposed on a doublet while the signal near 2.7 ppm for C-2 protons is comprised of two triplets. The signals for the monodeuterated product are shifted slightly upfield by 0.02 ppm. With both substrates, slightly more than half of the deuterium was retained in the product - 66% for the (2*R*)-[^{2}H] tyramine and 58% for the (2*S*)-[^{2}H] tyramine. This result can not be attributed to the nonstereospecificity of the amine substrates, since the chirality of the substrates was confirmed by reaction with *S*-(+)-*O*-acetylmandelic acid (see Figure 4.6 and discussion of the wash-out reaction). As well, the

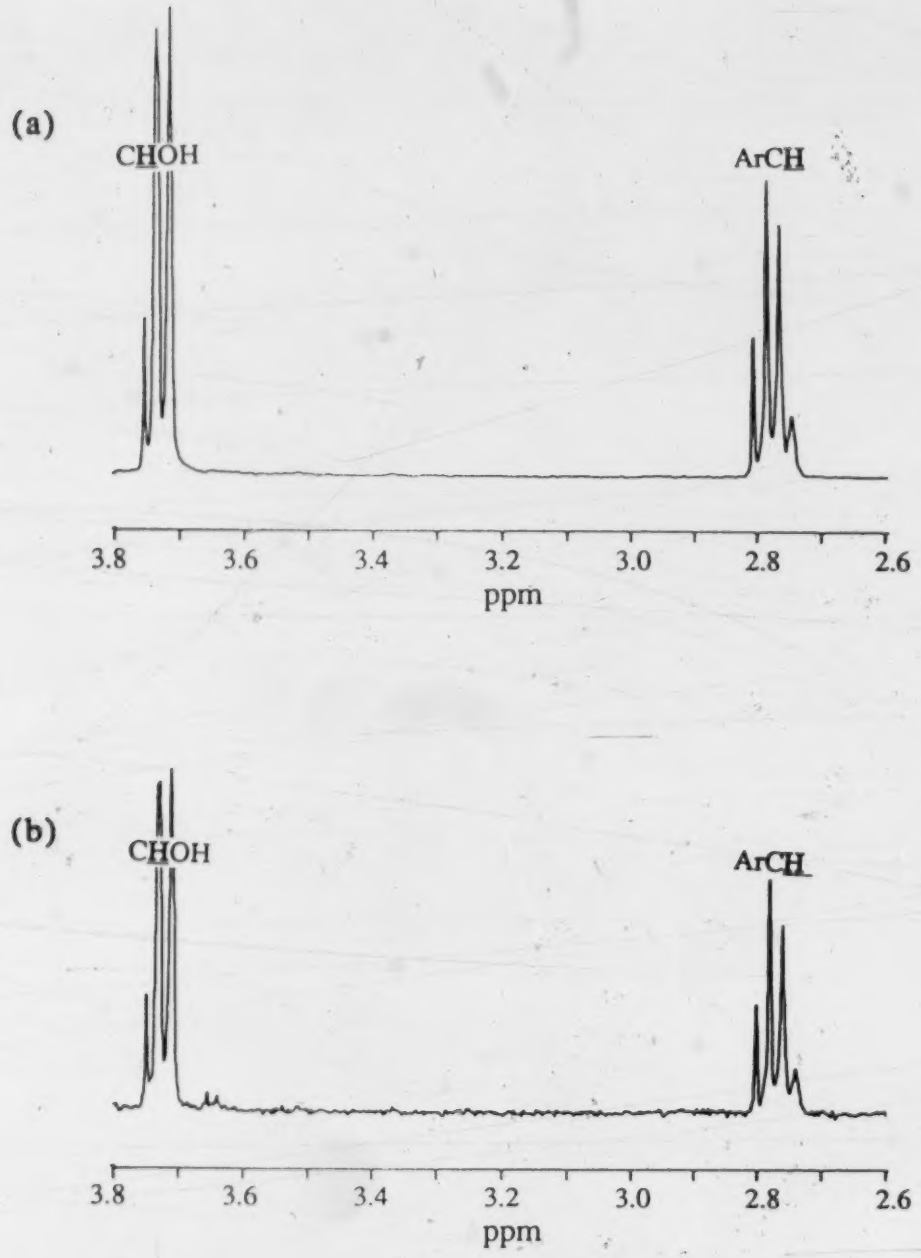


Figure 4.5. ^{1}H-NMR spectra of *p*-hydroxyphenethyl alcohols from coupled incubations of sheep plasma amine oxidase with (a) (2*R*)-[^{2}H] tyramine and (b) (2*S*)-[^{2}H] tyramine

effect is not due to different isoforms of the enzyme catalyzing opposite but stereospecific reactions since the reactions were carried out with purified isoforms. These isoforms were previously obtained with a combination of high and low ionic strength buffer elutions from anion exchange and HTP columns (Chapter 2).

The retention of greater than half of the deuterium suggests that an isotope effect of 1.9 for (2*R*)-[^{2}H] tyramine and 1.4 for (2*S*)-[^{2}H] tyramine is present for C-2 proton abstraction. This is calculated as the ratio of the monodeuterated product (deuterium retained) to the amount of diprotonated product (deuterium lost). In a truly nonstereospecific reaction with no isotope effect, 50% of the deuterium would have been lost from each substrate. Further work showing isotopic enrichment of product at low conversion rates, compared to high conversion rates, would offer additional support for the presence of an isotope effect. As well, the presence of a kinetic isotope effect on C-2 proton abstraction could be determined, using C-2 chiral amines and fully protonated amines as substrates. A possible isotope effect was observed during the wash-out reaction by the bovine plasma amine oxidase with tyramine (5). In this case the isotope effect for abstraction of the pro-*R* and pro-*S* proton was 3.8 and 1.6, respectively. Therefore, both the bovine and sheep plasma amine oxidase may have a larger isotope effect for the pro-*R* proton abstraction at C-2 while the opposite trend was found for C-1 proton abstraction (Chapter 3). The bovine plasma amine oxidase was found to catalyze a nonstereospecific proton abstraction from C-2 of dopamine (2). With this enzyme, 57% of deuterium was lost from (2*R*)-[^{3}H]-dopamine while 49% was lost from (2*S*)-[^{3}H] dopamine. It was suggested that this effect, however, was not due to an isotope effect, since the amount of tritium lost from the pro-*R* and pro-*S* position was approximately 100%. The presence or absence of an isotope effect on C-2 proton abstraction may depend on substrate orientation in the active site, and therefore may only be observed with tyramine.

The similarity between the stereochemistry of the sheep plasma amine oxidase in this work, and that reported for the bovine plasma amine oxidase (3), suggest that the active site of the two enzymes are alike. Therefore, the working model proposed for the bovine plasma enzyme of a syn relationship between proton abstraction at C-1 and C-2 with a single base being responsible for both proton abstractions (2, 15) may hold for the sheep enzyme.

Stereochemistry of Wash-In

Absolute Configurational Assignment of C-2 Protons

The extent of solvent incorporation in the wash-in experiments was determined from the ^{1}H-NMR spectra of the alcohol product, by integration of the signals attributed to monodeuterated and diprotonated species. However, in order to establish the stereospecificity of the wash-in reaction, a methodology was required to distinguish between the pro-*R* and pro-*S* protons at C-2 of tyramine. Previously, Klinman and coworkers (3) had utilized the specificity of dopamine-β-hydroxylase to determine the stereochemistry of the wash in reaction of bovine plasma amine oxidase. The alcohol products from the coupled amine oxidase-alcohol dehydrogenase reaction were chemically converted to amines, and then reacted with dopamine-β-hydroxylase. This enzyme is known to be specific for the pro-*R* hydrogen at C-2 of phenethylamines (9), and therefore, deuterium retained in products from the dopamine-β-hydroxylase reaction was assigned to the pro-*S* position. However, this method of configurational analysis was not efficient as product was lost during the chemical conversion and an additional work-up procedure was required to isolate the product from the dopamine-β-hydroxylase reaction.

In this work, a more direct method of determining the stereochemistry of the amine oxidase wash-in reaction was achieved by creating a chemically distinct environment for each proton at C-2 through the use of the chiral derivatizing agent, *S*-(+)-*O*-acetylmandelic acid (Scheme 4.5)[2] . Derivatization with an enantiomerically pure reagent at the C-1 hydroxyl creates nonequivalence of the protons at both C-1 and C-2. Each of these protons then exhibits a unique chemical shift in the NMR spectrum. Examples of chiral derivatizing agents includes camphanoyl chloride (16), Mosher's reagent (α-methoxy-α-(trifluoromethyl)phenylacetic acid (17), mandelic acid (8), methyl-2-hydroxy-2-phenethanoate or acetylmandelic acid (7). While such methodology has usually been commonly used to establish the absolute configuration of carbon atoms immediately adjacent to the derivatizing agent at C-1, it may also be used to distinguish between protons of the C-2 carbon (18, 19).

The method of derivatization and sample purification was simple and rapid, and could be completed in approximately three hours. As well, the reaction was ammenable to a small scale as typically one milligram of alcohol was used, although the reaction was equally successful when 0.3 to 0.5 milligrams of alcohol were used. Two molar equivalents of

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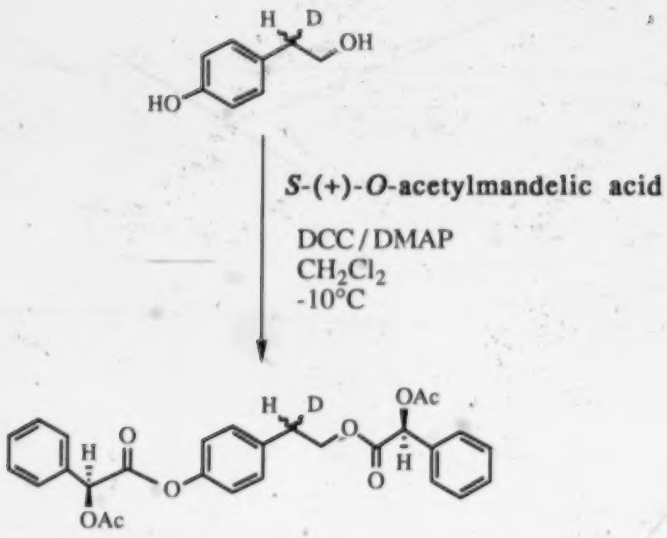
[2] Methodology suggested by Dr. John Beale, Dept. of Chemistry, University of Texas



Scheme 4.5. Formation of *S*-(+)-*O*-acetylmandelic ester with *p*-hydroxyphenethyl alcohol for configurational analysis of C-2 proton

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S-(+)-*O*-acetylmandelic acid and DCC were required as both the phenyl ring hydroxyl and the aliphatic C-1 hydroxyl were esterified. There was no evidence of preferential reactivity of one alcohol enantiomer compared to the other, similar to results reported for other aromatic and aliphatic alcohols (7, 8).

The methodology allowed us to confirm the stereospecificity of the bovine plasma amine oxidase wash-in reaction in protonated and deuterated buffers and to determine the absolute chemical shifts of the pro-*R* and pro-*S* protons at C-2 of the *S*-(+)-*Ø*-acetylmandelate ester . From the spectra of the ester derivatives of (2*R*)-[^{2}H] and (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohol the chemical shifts of the C-2 signal of the alcohol were found to be 2.32 ppm and 2.28 ppm respectively (Figure 4.6). Therefore the pro-*R* hydrogen resonated to high field of the pro-*S* hydrogen, as had been found for C-1 protons of a number of aliphatic and aromatic alcohols esterified with *S*-(+)-*O*-acetylmandelic acid (7). The signal for the single C-2 proton appeared to be a triplet, however, it is probable that it is composed of a pair of doublets with one peak superimposed on one another. The protons at C-1 and C-2 both become diastereotopic when the ester is formed (20) and the

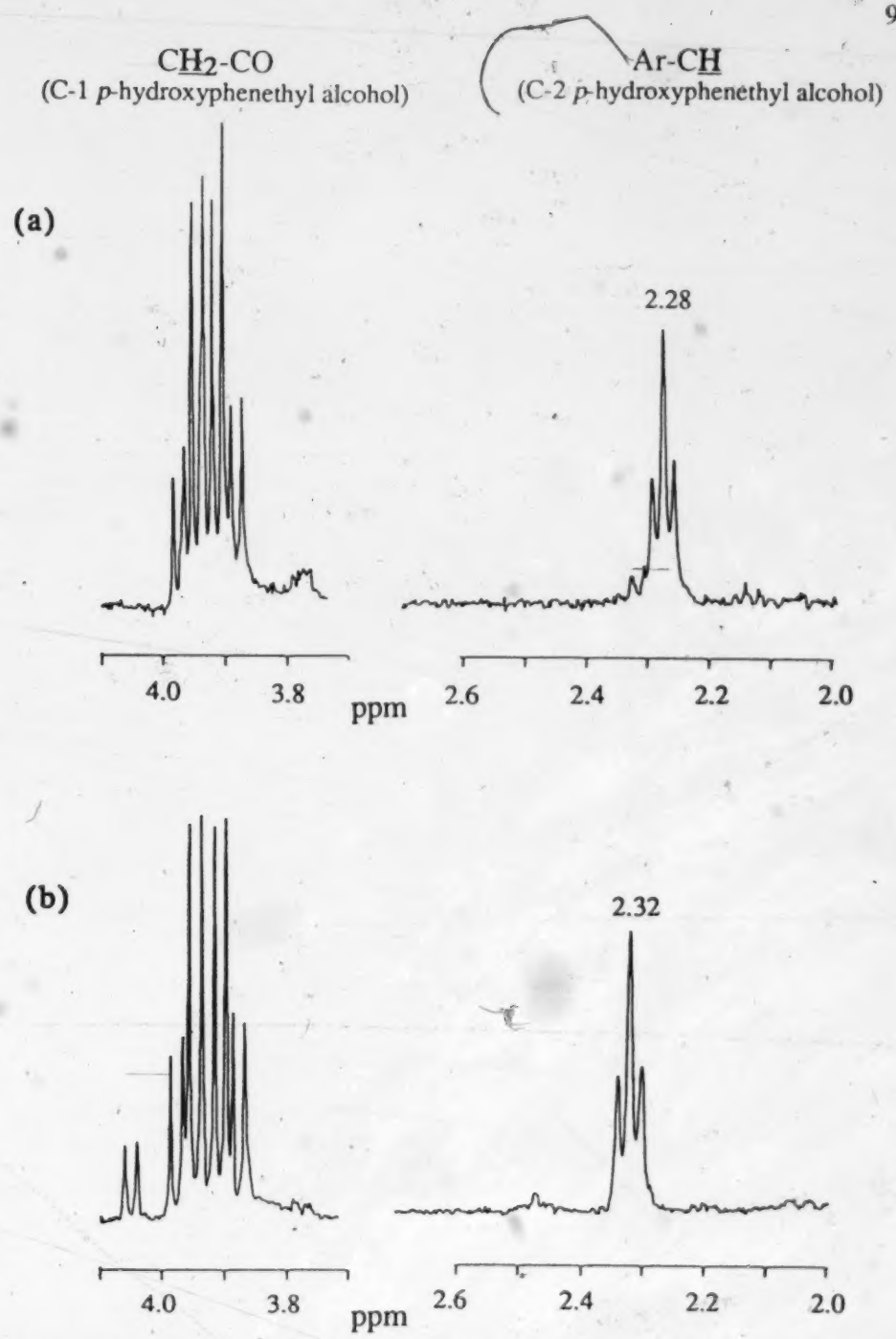

Figure 4.6. ^{1}H-NMR spectra of *S*-(+)-*O*-acetylmandelic acid derivatives with (a) (2*S*)-[^{2}H] *p*-hydroxyphenethyl alcohol and (b) (2*R*)-[^{2}H] *p*-hydroxyphenethyl alcohol

single proton at C-2 is coupled to each proton at C-1, creating a signal composed of two doublets. As well, a quartet is expected for each proton at C-1, resulting in an octet for the C-1 proton signal. This pattern was observed near 3.9 ppm for the C-1 protons, and illustrates the improved resolution of these C-1 protons, immediately adjacent to the chiral derivatizing agent, compared to the C-2 protons. Derivatives with the *R*-(-)-*O*-acetylmandelic acid were also prepared (spectra not shown). The ^{1}H-NMR chemical shifts for the C-2 signal of the pro-*R* and pro-*S* deuterated derivatives were 2.28 and 2.32 ppm respectively, which was the reverse of the *S*-(+)-*O*-acetylmandelic derivatives, as expected.

The ester derivative of [2,2-^{1}H] *p*-hydroxyphenethyl alcohol and [2,2,-^{2}H] *p*-hydroxyphenethyl alcohol were also obtained and the ^{1}H-NMR spectra are shown in Figure 4.7. The [2,2-^{2}H] *p*-hydroxyphenethyl alcohol was obtained by reacting [2,2-^{2}H]-tyramine with pea seedling amine oxidase in a protonated buffer. The pea seedling enzyme does not catalyze the solvent exchange pathway (1), allowing full retention of the deuterium at C-2 in the isolated alcohol product. The chemical shift of the C-2 protons from the [2,2-^{1}H]-alcohol derivative was 2.32 ppm, which confirms that the signals from the monodeuterated derivatives are shifted 0.02 ppm upfield due to the deuterium isotope effect. As expected, the dideuterated species lacks the signal for C-2 and exhibits a simpified signal for the C-1 protons near 3.9 ppm.

Although the signals for the pro-*R* and pro-*S* C-2 protons were partially resolved using a solvent of deuterated benzene and a 360 MHz NMR, some overlap was found to occur. An octet pattern was expected for each of the C-2 protons of the fully protonate derivative, while a total of only eight peaks for both protons was observed (Figure 4.7a). Attempts were made to improve the resolution of these signals using 500 mHz NMR and by obtaining the spectra in deuterated toluene, pyridine or nitrobenzene. While resolution of the C-1 protons was greatly improved on the 500 MHz NMR with benzene or on the 360 MHz NMR with nitrobenzene, no increase in resolution of the C-2 protons was obtained. The limited resolution of the signals from the C-2 protons increased the integration error slightly, however, the stereochemical assignment of the C-2 protons could clearly be made as pro-*R* or pro-*S* from the *S*-(+)-*O*-acetylmandelic ester.

Wash-In

The solvent wash-in behaviour of bovine and porcine SSAO and sheep plasma amine oxidase were determined by reacting [2,2-^{1}H] tyramine in deuterated buffer, or [2,2,^{2}H] tyramine in protonated buffer, with the coupling components to convert aldehyde products directly to alcohols. The alcohols were then derivatized with *S*-(+)-*O*

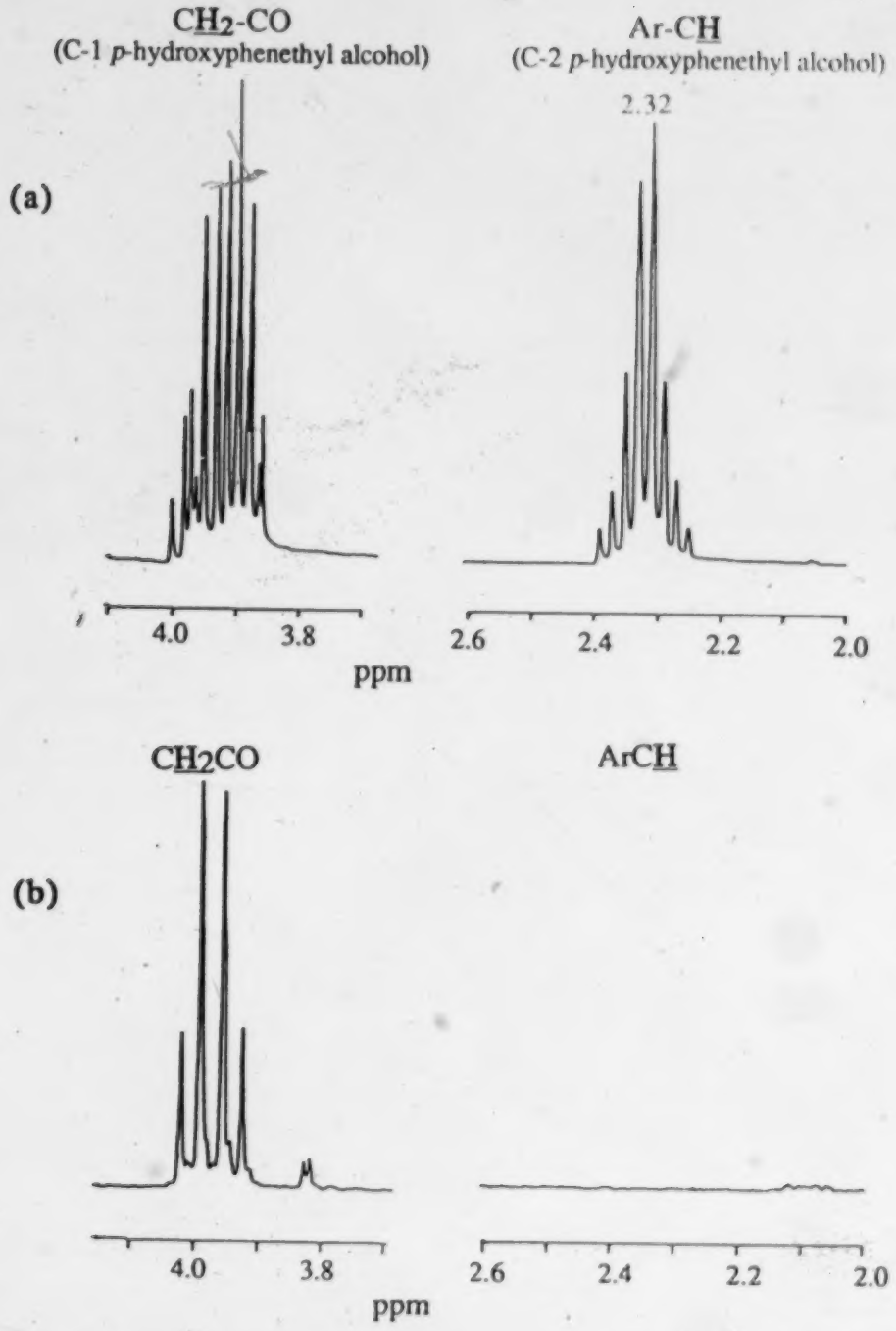

Figure 4.7. ^{1}H-NMR spectra of *S*-(+)-*O*-acetylmandelic acid derivatives with (a) [2,2-^{1}H] *p*-hydroxyphenethyl alcohol and (b) [2,2-^{2}H] *p*-hydroxyphenethyl alcohol

acetylmandelic acid, and the chemical shifts of the C-2 proton signals were compared to the standard (2*R*)-[^{2}H] or (2*S*)-[^{2}H] derivatives.

The wash-in reaction catalyzed by the bovine SSAO with deuterated buffer resulted in approximately 95% monodeuterated alcohol product with the remaining 5% being diprotonated (Figure 4.8a). This is seen on the ^{1}H-NMR spectra as a large doublet signal near 3.8 ppm for the C-1 protons superimposed on a minor triplet, and a large triplet near 2.7 ppm shifted slightly down field from a minor triplet. The larger signals are attributed to the monodeuterated species while the smaller signals shifted slightly downfield are attributed to the diprotonated signals The configurational analysis of the C-2 protons indicated that wash-in had occurred in the pro-*R* position, resulting in (2*R*)-[^{2}H] derivative with a chemical shift for this proton of 2.32 ppm (Figure 4.8b).

Alternatively, the alcohol products of the bovine wash-in reaction with protonated buffer and [2,2,-^{2}H] tyramine were found to be 6 % diprotonated and 92% monodeuterated and 2% dideuterated (Figure 4.9a). Configurational analysis of the alcohol products indicated that the monodeuterated species was the (2*S*)-[^{2}H] ester derivative with a chemical shift for the C-2 proton signal of 2.28 ppm, indicating pro-*R* wash-in (Figure 4.9b). As well, the small amount of diprotonated product was present as an ester derivative, as was observed in the alcohol.

The presence of the diprotonated alcohol indicates that the reaction in protonated buffer occurs somewhat differently than in deuterated buffer. The small amount of dideuterated product was expected, as this corresponds to product formed by by-passing the imine-enamine pathway, as was found in the reaction with the deuterated buffer. However, the diprotonated alcohol indicates a greater extent of solvent wash-in at C-2 than seen with the alternate reaction carried out in deuterated buffer. The diprotonated species could arise from a slight uncoupling of the alcohol dehydrogenase reaction, allowing an abstraction and reprotonation at C-2 could create a small amount of the diprotonated alcohol product. It is not clear why the coupling of aldehyde to alcohol should be less efficient in this experiment. Alternatively, two deuterium abstractions from the pro-*R* and pro-*S* positions of C-2, with reprotonation from solvent in each case, would yield the diprotonated product at C-2. Since a small amount an alternate stereochemical mode was found to occur during proton abstraction at C-1 and in the wash-out reaction, a similar amount of pro-*S* proton abstraction might occur during the wash-in reaction, and may account for the formation of the diprotonated product.

The wash-in reaction of the porcine SSAO was very similar to that of the bovine SSAO. The reaction in deuterated buffer with [2,2-^{1}H]-tyramine yielded 95%

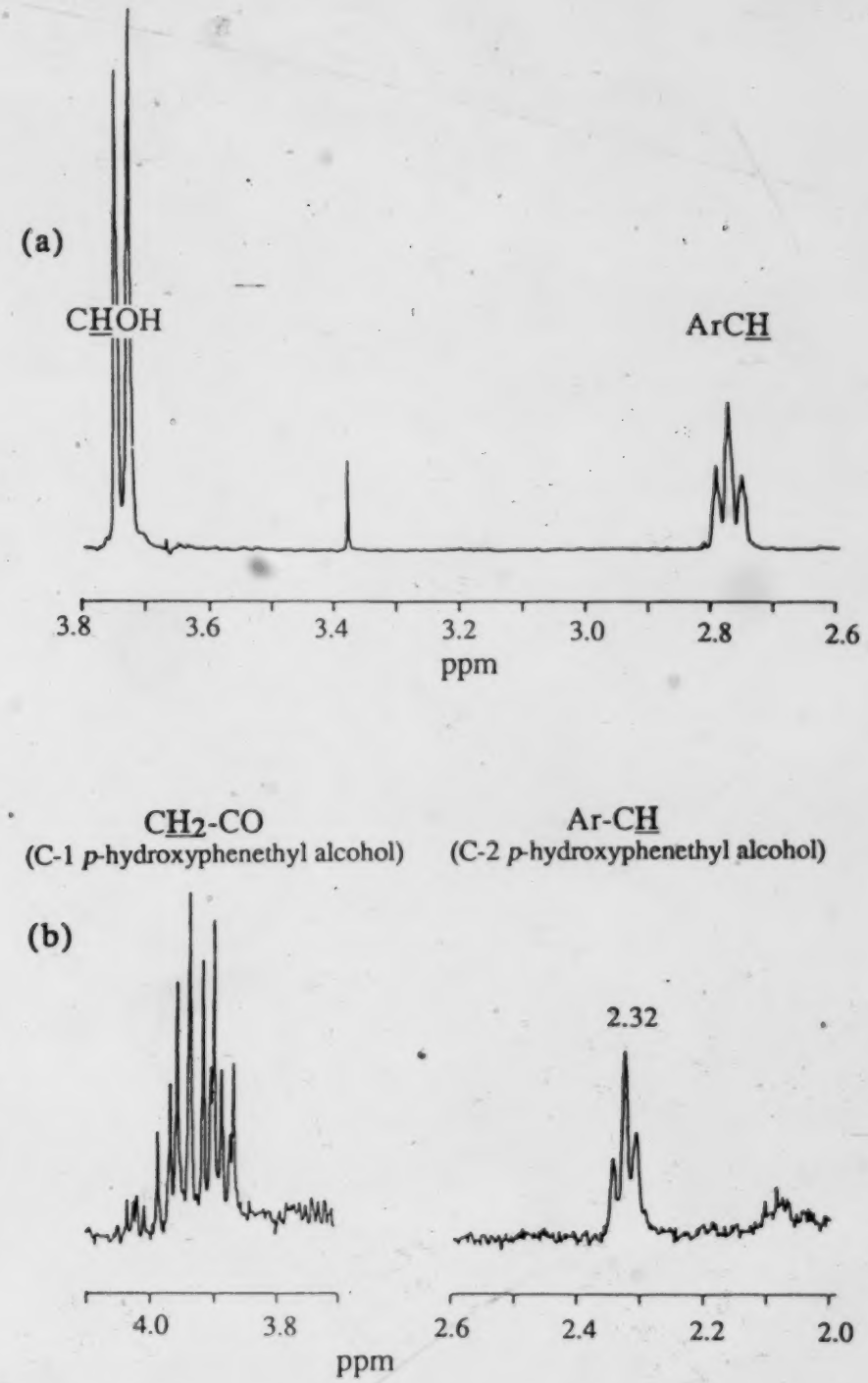

Figure 4.8. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of bovine SSAO with [1,1-^{1}H] tyramine in $^{2}H_2O$ and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

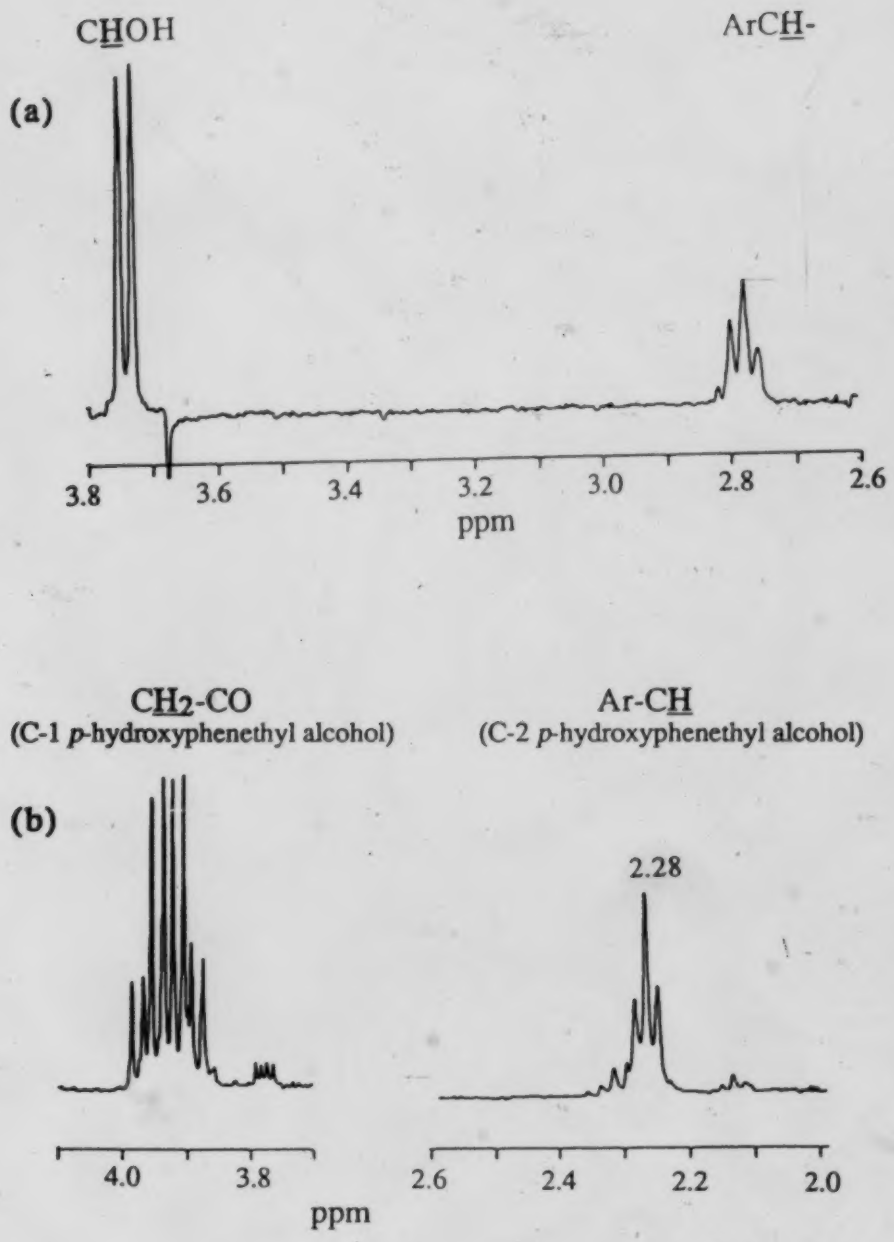

Figure 4.9. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of bovine SSAO with [1,1-^{2}H] tyramine in H_2O and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

monodeuterated alcohol product, with the residual product being diprotonated (Figure 4.10a.) Again, as with the bovine SSAO, configurational analysis indicated that all the monodeuterated alcohol was due to wash-in of deuterium to the pro-*R* position. The chemical shift of the ^{1}H-NMR signal for the C-2 protons was 2.32 ppm (Figure 4.10b), as seen for the (2*R*)-[^{2}H] standard.

The protonated buffer wash-in reaction catalyzed by the porcine SSAO yielded 12% dideuterated, 85% monodeuterated and 9% diprotonated alcohol product (Figure 4.11a). This suggested that the reaction with the dideuterated substrate was less stereospecific than with the protonated substrate as was observed with the bovine SSAO. None the less, the C-2 ^{1}H-NMR signals from the *S*-(+)-*O*-acetylmandelic ester of the monodeuterated alcohol occurred at a chemical shift of 2.28 ppm (Figure 4.11b), indicating that it was the (2*S*)-[^{2}H] derivative, and that wash-in had been pro-*R*, as expected.

For both the bovine and porcine SSAO, the wash-in reaction in protonated and deuterated buffer occurred with pro-*R* specificity, just as wash-out had been pro-*R*. This suggests that wash-in and wash-out occur from the same side of the imine double bond and that the two processes may be microscopically reversible, unlike the reaction catalyzed by the bovine (3), porcine (5) and sheep plasma amine oxidase (see following discussion) (Table 4.1). The products obtained from the wash-in incubations suggest, however, that the reaction in protonated and deuterated buffer do not occur equivalently. This effect may be due to an isotope effect on the wash-out reaction as the bovine SSAO was found to retain a significant amount of deuterium (30%) from the (2*R*)-[^{2}H] tyramine in the wash-out reaction. However, this effect was not noted for the porcine SSAO.

The sheep plasma amine oxidase catalyzed stereospecific the pro-*R* wash-in of solvent to C-2 of tyramine. The alcohol products from the deuterated buffer wash-in were

Table 4.1. Stereochemistry of Solvent Exchange Reaction

Enzyme Source	C-2 Solvent Exchange	
	In	Out
Porcine Aorta [a]	pro-*R*	pro-*R*
Bovine Aorta [a]	pro-*R*	pro-*R*
Porcine Plasma [a, b]	pro-*R*	Nonstereospecific
Bovine Plasma [b, c]	pro-*R*	Nonstereospecific
Sheep Plasma [a]	pro-*R*	Nonstereospecfic

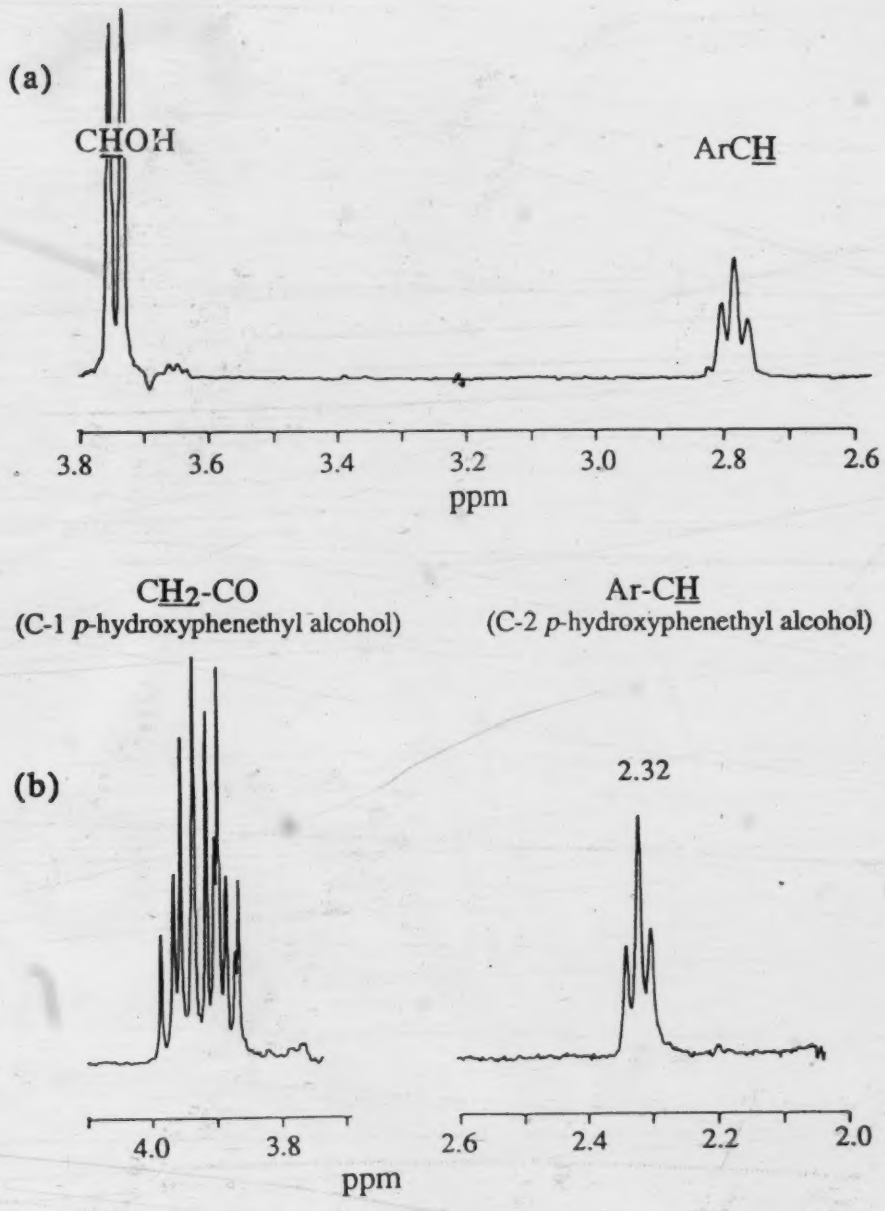

Figure 4.10. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of porcine SSAO with [1,1-^{1}H] tyramine in $^{2}H_2O$ and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

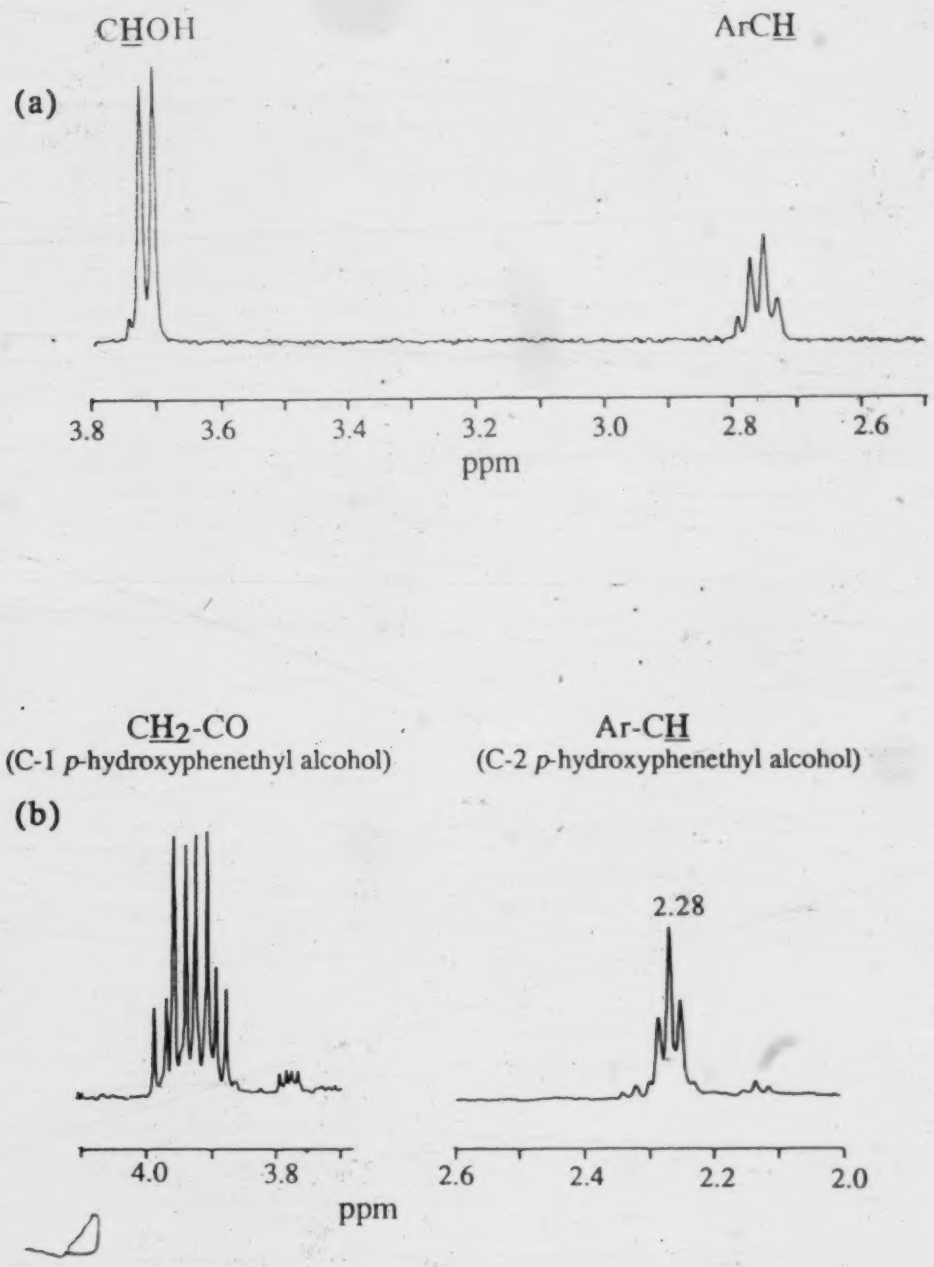

Figure 4.11. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of porcine SSAO with [1,1-^{2}H] tyramine in H_2O and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

predominantly monodeuterated (94%), with the remaining material being diprotonated (Figure 4.12a.) Configurational analysis indicated that the monodeuterated species was deuterated in the pro-*R* position, as the chemical shift for the C-2 signal was 2.32 ppm (Figure 4.12b). This implies that wash-in was to the pro-*R* position.

The products of the corresponding wash-in reaction in protonated buffer were 60% monodeuterated, 30% dideuterated and 10% diprotonated alcohol (Figure 4.13a). The C-1 and C-2 proton signals for the monodeuterated product were attributed to the large doublet and triplet signals near 3.8 and 2.7 ppm, respectively. The minor signals shifted slightly downfield at the same ppm arose from the diprotonated alcohol. The signal from the dideuterated product was a singlet which is superimposed on the upfield peak of the doublet near 3.8 ppm. Configurational analysis of the product indicated that the monodeuterated species was the (2*S*)-[^{2}H] ester derivative, as a chemical shift of 2.28 ppm was obtained for the C-2 proton signal (Figure 4.13b). Again, this implies that the wash-in reaction was predominantly pro-*R*, and that reprotonation occurred from only one side of the imine double bond. It is interesting that less stereospecificity was also observed for the wash-in reaction in protonated buffer compared to deuterated buffer for the bovine plasma amine oxidase, while generating the C-2 chirally deuterated alcohols. A considerable amount of the product of the protonated buffer wash-in reaction was the dideuterated alcohol, obtained by bypassing the solvent exchange pathway. These results indicate that the expression of the imine:enamine pathway is altered by experimental conditions, and may be due to an isotope effect on wash-out from the pro-*R* position at C-2.

The stereochemical inconsistency of the wash-out (nonstereospecific) and wash-in (pro-*R*) reactions for the sheep plasma amine oxidase implies that the two processes are not the reverse of each other. A similar result was reported for the bovine and porcine plasma amine oxidase reaction (3, 5). It is difficult to account for this lack of reversibility based on our current knowledge of the mechanism of these enzymes. An understanding of these processes will require additional information concerning the active site residues and their orientation.

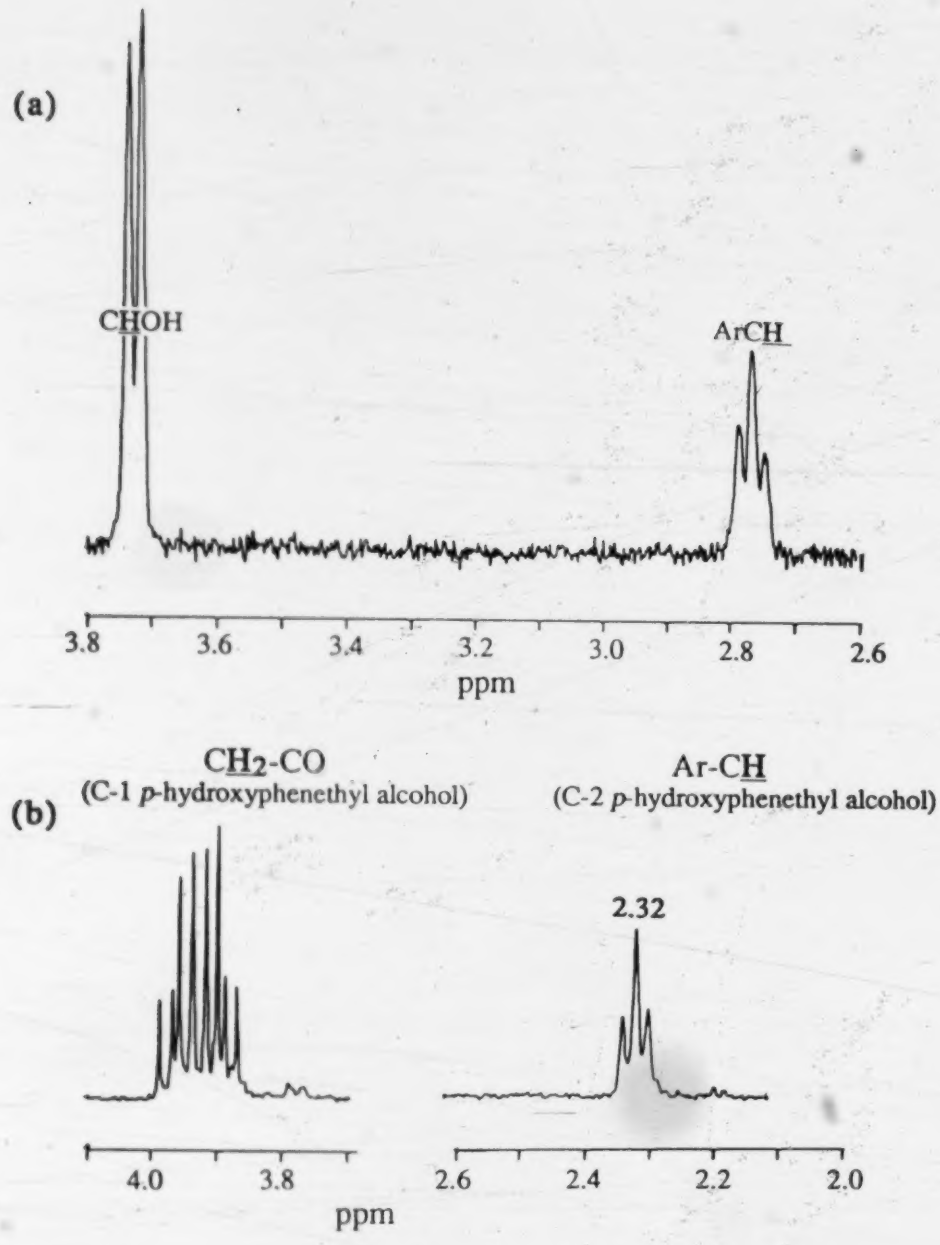

Figure 4.12. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of sheep plasma amine oxidase with [1,1-^{1}H] tyramine in $^{2}H_2O$ and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

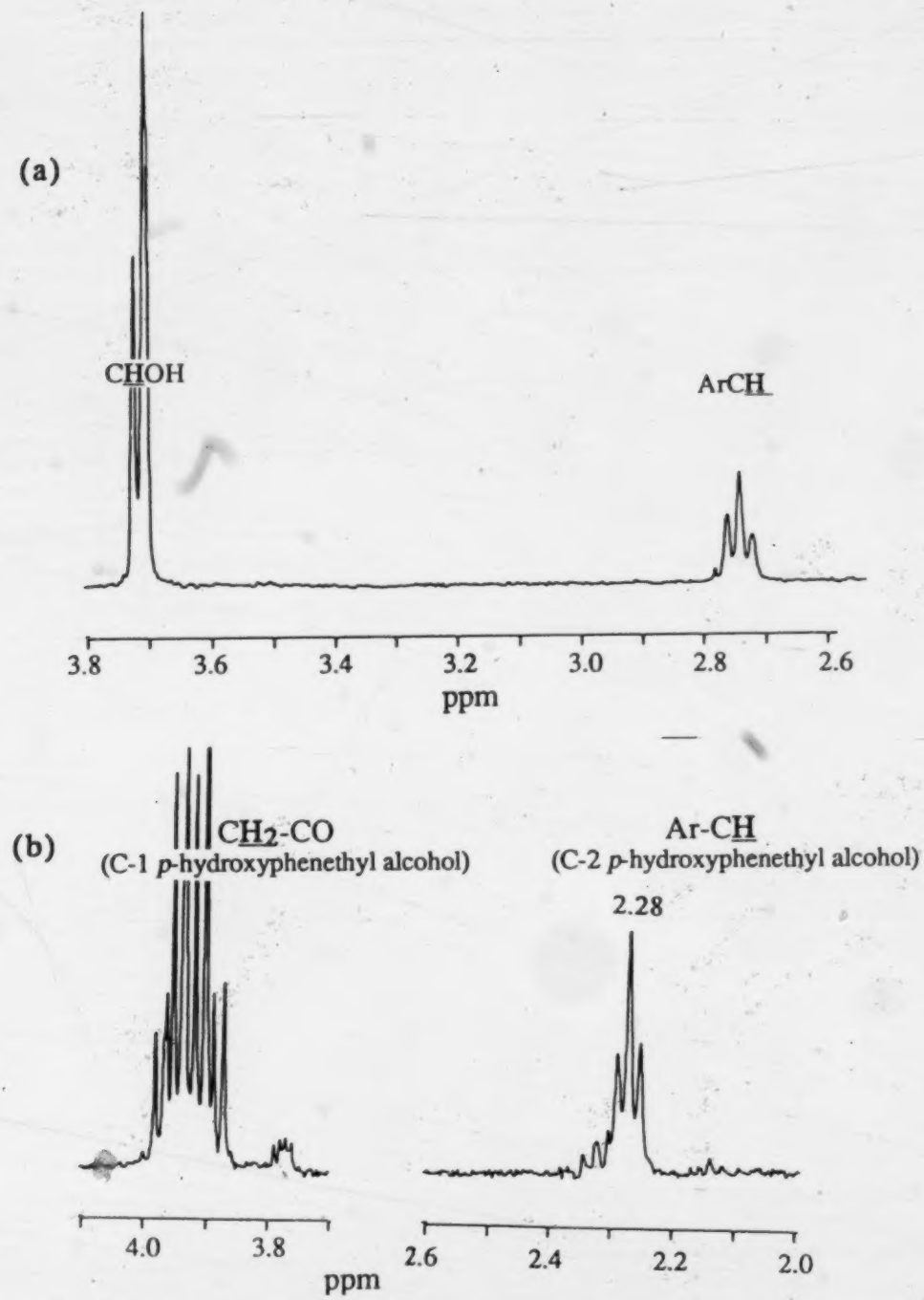

Figure 4.13. ^{1}H-NMR spectra of (a) *p*-hydroxyphenethyl alcohol derived from coupled incubation of sheep plasma amine oxidase with [1,1-^{2}H] tyramine in H_2O and (b) *S*-(+)-*O*-acetylmandelic acid derivative of the alcohol

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Chapter 5. Conclusions of This Study

This work has reported on the isolation, partial physio-chemical characterization and stereochemical evaluation of the semicarbazide sensitive amine oxidases (SSAO) from bovine and porcine aortic tissue. The characteristics of the SSAO were compared to those of two copper amine oxidases from porcine kidney and sheep plasma, also isolated for this work, and to reports of other copper amine oxidases. Several lines of investigation suggest that the SSAO constitute a class of amine oxidase that is distinct from the copper amine oxidases.

Physio-chemical Properties

The SSAO are membrane bound enzymes, in contrast to the soluble nature of the copper amine oxidases, and showed a high affinity for benzylamine and aliphatic monamines. The solution of the enzyme did not display the characteristic pink or peach chromophore which is typical of the copper amine oxidases and regarded as a physical manifestation of the organic cofactor. As well, the reactivity of the enzyme with semicarbazide and phenylhydrazine suggested that the cofactor was a quinone type molecule but with properties different from TOPA, the cofactor of some copper amine oxidases (1). Further investigation into the nature of the cofactor of the SSAO should be carried out, as it is potentially a yet unidentified cofactor in mammalian biochemistry. This could be accomplished by labelling the cofactor with radioactive carbonyl reagent, isolating a peptide containing the label, and identifying the residues and cofactor using a direct technique such as mass spectroscopy.

Stereochemistry of Tyramine Oxidation

The SSAO from bovine and porcine aorta was found to catalyze the abstraction of the pro-*S* proton from C-1 of tyramine, as was observed for the copper containing porcine kidney amine oxidase. This stereochemistry is consistent with that reported for another SSAO enzyme from rat aorta (2), and suggests that the mechanism of oxidation for the aortic enzymes has been conserved across animal species. This is in contrast to the diverse stereochemical patterns of C-1 proton abstraction exhibited by the copper amine oxidases from a common tissue, such as plasma. It would be of interest to examine the stereochemistry of SSAO from alternate tissues, such as brown adipose tissue or dental pulp, to establish if these also followed pro-*S* stereochemistry at C-1.

A sheep plasma enzyme preparation with a high specific activity, and two major isoforms of the enzyme catalyzed an apparent nonstereospecific proton abstraction from C-

1, as had been reported for a low specfic activity preparation (3). This stereochemistry is similar to that observed for the bovine plasma amine oxidase, and is attributed to the existence of dual binding modes for tyramine, each with opposite but absolute stereospecificity (4). This confirms that the apparent nonstereospecifity is an intrinsic property of the enzyme, and not due to the presence of two amine oxidase activities with opposite stereochemistries.

It might be suggested that the enzymes, such as the bovine or sheep plasma amine oxidase, that exhibit dual binding modes for phenethylamines, are the evolutionary forerunners of the enzymes with a single binding mode. There should not be a metabolic influence on the evolution of proton abstraction from C-1 of an amine since the aldehyde becomes pro-chiral at this carbon (i.e. the reaction is stereochemically cryptic to the cell (5)). However, the efficiency of the enzyme reaction itself may be enhanced by evolving to a single binding mode and the optimization of it alone. Experimental data from the dual binding mode enzymes already suggests that the pro-S proton abstraction is the preferred mode since the associated isotope effect is larger than that for pro-R proton abstraction. This has been interpretted as the pro-S mode having a preferred orientation in the active site of the enzyme, allowing the C-H bond cleavage to be more rate limiting. The evolution of an enzyme with only the pro-S binding may be an extension of this. The pro-R specificity of the porcine plasma enzyme does not support this, however (6). It would be of interest to compare the amino acid sequences of the various copper and SSAO enzymes to evaluate their evolutionary relatedness.

Solvent Exchange Reaction

While pro-*S* specificity at C-1 does not distinguished the SSAO from the copper amine oxidases, the observation that SSAO also catalyze the solvent exchange pathway, does. It was found that both proton abstraction and reprotonation at C-2 of tyramine occured with pro-*R* specificity. This suggests that wash-out and wash-in take place on the same side of the imine double bond and may be microscopically reversible processes. This has not been the case with the copper amine oxidases which catalyze the solvent exchange pathway, including the sheep plasma amine oxidase. These enzymes, despite the heterogeneity of proton abstraction at C-1, were all found to catalyze the pro-*R* proton abstraction from C-2 followed by nonstereospecific reprotonation.

This work has, then, provided only a preliminary insight into the mechanism of the SSAO enzymes. Further work, including the pH dependence of catalysis and isotope effects, would aid in establishing if the proton abstraction at C-1 and C-2 is base-catalyzed,

and if so, by a single base or two bases. As well, there is a need to establish if the SSAO enzymes follow a transaminase type mechanism, as suggested for some copper amine oxidases. This could be confirmed by determining the position of ammonia release, relative to that for aldehyde.

As more mechanistic information becomes available for these enzymes, it is likely that inhibitors specific for SSAO can be developed. These in turn may aid in determining the physiological role of the enzyme.

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Appendix I

Synthesis of (1R)-[^{2}H] and (1S)-[^{2}H] Tyramine

DL-[2-^{2}H]tyrosine was obtained by reacting fully protonated tyrosine with KO^{2}H in ^2H_2O in the presence of pyridoxal hydrochloride (1). After neutralization of the reaction, the deuterated amino acid was isolated as a solid and recrystallized from hot water by the addition on ethanol. The extent of deuteration at C-2 position was greater than 95% as determined by ^{1}H-NMR spectroscopy and isolated yields for the reaction were 20 - 30%. (1*S*)-[^{2}H] Tyramine was obtained by decarboxylation of **DL**-[2-^{2}H]tyrosine with *Streptococcus faecalis* tyrosine decarboxylase in 50 mM sodium acetate buffer, pH 5.8. The corresponding (1*R*)-[^{2}H] tyramine was obtained by decarboxylation of **L**-[2-^{1}H]tyrosine with the same enzyme, with the reaction carried out in deuterated sodium acetate buffer, pD 6.0, where pD is the uncorrected pH meter reading. For both reactions, product amines were separated from unreacted amino acid by chromatography on Amberlite IRC 50 (H+ form). Unreacted amino acid was eluted with water and amine was eluted with 1M acetic acid. Amine was neutralized with 0.2 N HCl and evaporated to dryness.

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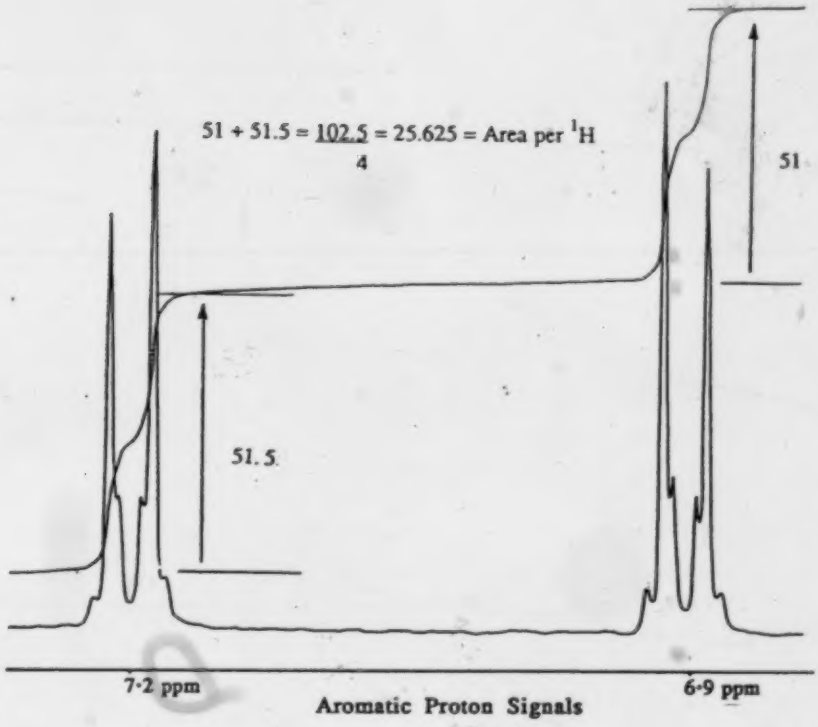
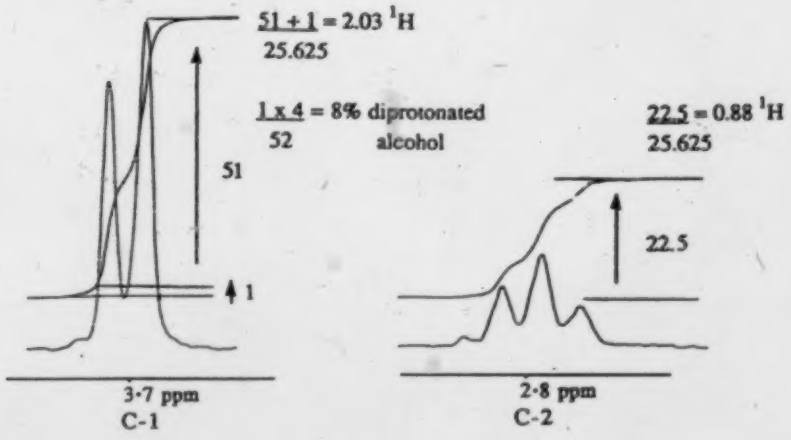
Appendix II

Calculation of Percent of C-1 Monoprotonated, Dideuterated and Diprotonated Alcohol Products from ^{1}H-NMR Spectra





(a) Calculation of # of Protons at C-1 and C-2

Peak areas were calculated from the integration of the signals for C-1 (3.8 ppm), C-2 (2.7 ppm) and the aromatic protons (6.9 and 7.1 ppm). The total aromatic protons, 4, was used to calibrate the number of area units per proton, and the number of protons at C-1 and C-2 were calculated.

(b) Calculation of Diprotonate Species

The signals corresponding to a doublet at C-1 and a triplet at C-2, shifted 0.02 ppm upfield were assigned to the monoprotonated species. The single peak downfield from the C-1 signals was considered to be the leading peak from a triplet from protonated alcohol, and therefore the area of this leading peak x 4 = area of triplet (peak ratio 1:3:1).The ratio of the area of the triplet to the area of the entire signal for C-1 yields an estimate of the percent diprotonated product.

(c) Calculation of Monodeuterated and Dideuterated Species

From the integration at C-2, the total number of protons can be calculated as described above. The molecule has two positions at this carbon, and therefore the number deuterium atoms = 2 - # protons. The amount of monodeuterated and dideuterated alcohol is calculated by completing the following table:

Species	Total # H	Total # D	% of each species
HH	2 x .08 = .16	(n.c.)*	8
HD	0.88-0.16 = 0.72	0.72	72
DD	(n.c.)*	1.12 - 0.72 = 0.40	20
Total	0.88	1.12	100

* (n.c.) - no contribution